PROTECTIVE EFFECT OF AQUEOUS EXTRACTS OF *AFRAMOMUM LONGISCAPUM* SEED AGAINST SODIUM ARSENITE - INDUCED HEPATOTOXICITY AND CLASTOGENICITY IN WISTAR RATS

BY

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MARCH, 2014

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CERTIFICATION

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DEDICATION

I dedicate this piece of work to God Almighty, the I am that I am.

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ABSTRACT

The use of medicinal plants has been found to be effective in the treatment of diseases such as cancer. *Aframomum longiscapum* seed has been used in the treatment of cancer in ethno medicine but has not been subjected to appropriate scientific evaluation. This study was therefore designed to evaluate the protective effect of Aqueous Extract of *Aframomum longiscapum* (AEAL) seed against sodium arsenite- and ethanol-induced hepatotoxicity and clastogenicity in male Wistar rats.

Phytochemical composition of dried milled seeds of *Aframomum longiscapum* was evaluated using standard procedures. Twenty five male albino Wistar rats (150-250 g) were randomly divided into five groups of five rats each: Group I (control) - distilled water, Group II- sodium arsenite (2.5mg/kg), Group III - sodium arsenite (2.5mg/kg) in 3% ethanol (v/v), Group IV- AEAL (122.5 mg/kg), Group V- sodium arsenite (2.5mg/kg) in 3% ethanol + AEAL (122.5mg/kg body weight). After five weeks of treatment, hepatotoxicity was assessed using serum activities of Alkaline Phosphatase (ALP), Alanine amino Transferase (ALT), Gamma Glutamyl Transferase (GGT) and Aspartate amino Transferase (AST) by spectrophotometry. Clastogenicity was determined using bone marrow cytology to identify Micronucleated Polychromatic Erythrocytes (MnPCEs) by microscopy. Sperm counts, motility, viability and morphological abnormalities were estimated using microscopic techniques. Histology was carried out using Haematoxylin and Eosin. Data were analysed using Student's t test at p = 0.05.

Phytochemical screening revealed the presence of alkaloids, saponins, flavonoids and cardenolides. There were increased levels of liver enzymes on exposure to sodium arsenite compared with the control (ALP = 236.8 ± 15.2 vs 432.2 ± 41.4, ALT = 23.1 ± 5.9 vs 29.9 ± 2.8, GGT = 2.6 ± 1.9 vs 4.6 ± 1.6 and AST = 47.5 ± 9.2 vs 66.0 ± 15.6 U/L). The elevated levels of these enzymes were significantly reduced by about 2 folds in AEAL–treated rats compared with the sodium arsenite group (29.9 ± 2.8 vs 19.8 ± 3.1). The degree of reduction of MnPCEs was 2 folds in the treated-animals (15.4 ± 5.2 vs 26.5 ±2.1). The AEAL reversed the severe hepatic degeneration and necrosis induced by sodium arsenite and caused a significant decrease in sperm counts (126.1 ± 8.5 vs 85.5 ± 14.9) and motility (90.0 ± 10.0 vs 52.5 ± 15.0). However, there were significant increases in sperm viability (92.5 ± 3.5 vs 93.3 ± 4.0) and morphological abnormalities (10.1 ± 1.9 vs 13.4 ± 2.4).

Aframomum longiscapum seed extract has protective effect against sodium-arsenite induced hepatotoxicity and clastogenicity in intoxicated rats. The extract however had deleterious side effects on male fertility in the treated rats. This plant extract should be administered with caution to human subjects given the provisional nature of these data.

Key words: *Aframomum longiscapum*, Sodium arsenite-induced hepatotoxicity, Male fertility

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

Cells can experience uncontrolled growth if there are damages or mutations to DNA. Therefore, damage to the genes during cell division can lead to uncontrollable cell growth. Four key types of genes are responsible for the cell division process: oncogenes give instruction to cells when to divide, tumour suppressor genes give command to cells when not to divide, suicide genes control apoptosis and tell the cell to kill itself if something goes wrong, and DNA-repair genes instruct a cell to repair damaged DNA. Cancer occurs when the cell's gene mutations make the cell unable to correct DNA damage and unable to commit suicide. Similarly, cancer is a result of mutations that inhibit oncogenes and tumour suppressor genes function, leading to uncontrollable cell growth. Carcinogens are a class of substances that are directly responsible for damaging DNA, promoting or aiding cancer. Examples of carcinogens include tobacco, asbestos, arsenic, radiation such as gamma and x-rays, the sun, and compounds in car exhaust fumes. When our bodies are exposed to carcinogens, free radicals are formed that try to steal electrons from other molecules in the body. These free radicals initiate series of reactions leading to the damage of cells and thereby affecting their ability to function normally.

Inorganic arsenic compounds are widely distributed in nature and a lot of epidemiological evidence exists, associating occupational and environmental exposure to them with human carcinogenesis (IARC 1973, 1980; Arteel *et al.,* 1981). For instance, exposure to trivalent and pentavalent forms of arsenic, which occurs worldwide primarily through occupational and environmental exposure, causes characteristic skin alterations (ulcetration), including hyperkeratosis and skin cancer (Yoshida *et al*., 2004). Epidemiological studies conducted in Taiwan (Chiou *et al*., 1995), Argentina (Smith *et al*., 1996), Chile (Smith *et al*., 1998), and Japan (Tsuda *et al.,* 1995) indicated a connection between arsenic exposures from contaminated drinking water among the inhabitants. It is also known that arsenic interact with other substances, metals inclusive there by potentiating its effects or vice versa (Odunola *et al.*,2007). There is growing evidences that sodium arsenite intoxication can

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compromise the integrity of the liver in mouse, rat, fish, and goat (Sharma *et al*., 2009; Roy *et al*., 2009; Srinivas *et al*., 2007). Recently some studies suggest the use of antioxidants and antioxidant rich foods and herbal medicinal plant for the management of arsenicosis (Das *et al.,*2010).

All cancers begin in cells, the body's basic unit of life. To understand cancer, it's helpful to know what happens when normal cells become cancer cells. The body is made up of many types of cells. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. When cells become old or damaged, they die and are replaced with new cells. However, sometimes this orderly process goes wrong. The genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division. When this happens, cells do not die when they should and new cells form when the body does not need them. The extra cells may form a mass of tissue called a tumour.

Induction of cancer is frequently associated with DNA damage, changes in ploidy of cells, and non-random chromosome aberrations which can result from exposure to arsenic (Sinha *et al.,* 2005). Association between chronic alcohol abuse and the development of cirrhosis, as well as between cirrhosis and the development of hepatocellular carcinoma (HCC), is well documented (El-Serag, 2001; Lonnecker, 1995). Acute and chronic ethanol treatment has been shown to increase the production of reactive oxygen species, lower cellular antioxidant levels, and enhance oxidative stress in many tissues, especially the liver (Aparajita and Cederbaum, 2006). Ethanol-induced oxidative stress plays a major role in the mechanisms by which ethanol produces liver injury (Aparajita and Cederbaum, 2006).

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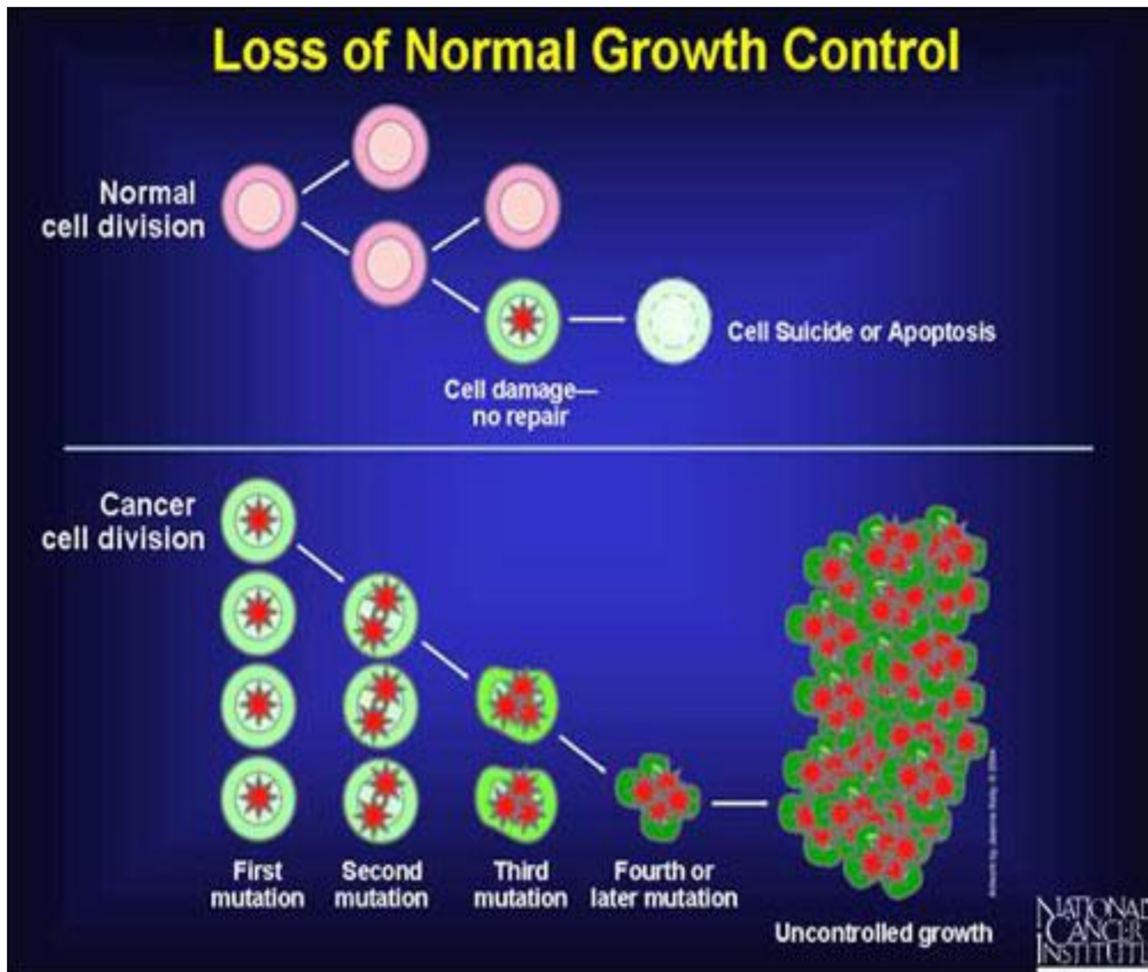

Figure 1.1: Diagram showing loss of normal cell growth control

The medicinal use of certain plants has been almost as important to man as their food uses (Muhammad and Amusa, 2005). Men learnt to exploit plants for medicine, almost as early as they cultivated them for food. However, Man has learned over the years, the art of treating diseases with the use of natural products, within his immediate environment (Cragg and Newman, 2001). Natural products from higher plants have indeed offered humanity, clinically useful drugs, such as reserpine, vincristine, physostigmine, quinine, d-tubocurarine, ginseng, and artemisinin (Soejarto, 1996). Nigerian flora has contributed immensely to the health care of the rural populace, who has been using many indigenous plants, to cure their ailments since time immemorial (Abila *et al*., 1979; Gill, 1992; Akah and Nwambie, 1994). However, little is known about the pharmacological efficacy of these herbal plants (Gill, 1992). *Aframomum longiscapum* Schumann (Zingiberaceae) is one of such notable medicinal plants, popularly used in Nigeria and other countries in the world as a spice. Although so much work has been carried out on the chemopreventives due to its potent anti-oxidant activity, as it significantly reduced the levels of malonydialdehyde (MDA) formation in rat RBC exposed to phenylhydrazine properties of the plants like *Aframomum melegueta* (Solomon and Babatunde, 2007), and *Aframomum danielli* (Fasoyiro and Adegoke, 2007) , non has so far being carried out on the possible counteracting effect of *Aframomum longiscapum* Schumann (Zingiberaceae) on sodium arsenite and ethanol-induced toxicities. The present study was undertaken to evaluate the protective effects of aqueous extract of *Aframomum longiscapum* seed on sodium arsenite and ethanol-induced hepatotoxicity and clastogenicity in male Wister albino rats.

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1.1 AIM AND OBJECTIVES

The aim of this research work is to determine the protective effect of aqueous extracts of *Afromomum longiscapum* seed on sodium arsenite and ethanol-induced hepatotoxicity and clastogenicity in male Wistar albino rats and also:

- To determine the phytochemical profile of *Afromomum longiscapum* seed
- To determine its effect on biochemical liver enzyme profiles
- To determine its effect on hepatocyte integrity and histopathology
- To determine its potential anticlastogenic effects
- To determine its effect on male fertility

1.2 LITERATURE REVIEW

1.2.1 ETIOLOGY OF CANCER

Cancer is ultimately the result of cells that uncontrollably grow and do not die. Normal cells in the body follow an orderly path of growth, division, and death. Programmed cell death is called apoptosis, and when this process breaks down, cancer begins to form. Unlike regular cells, cancer cells do not experience programmatic death and instead continue to grow and divide. This leads to a mass of abnormal cells that grows out of control. Cancer harms the body when damaged cells divide uncontrollably to form lumps or masses of tissue called tumours (except in the case of leukaemia where cancer prohibits normal blood function by abnormal cell division in the blood stream). Tumours can grow and interfere with the digestive, nervous, and circulatory systems, and they can release hormones that alter body function. Tumours that stay in one spot and demonstrate limited growth are generally considered to be benign. More dangerous, or malignant, tumors form when two things occur:

1. A cancerous cell manages to move throughout the body using the blood or lymph systems, destroying healthy tissues in a process called invasion.
2. That cell manages to divide and grow, making new blood vessels to feed itself in a process called angiogenesis.

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There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. The five broad groups that are used to classify cancer are:

1. Carcinomas are characterized by cells that cover internal and external parts of the body such as lung, breast, and colon cancer.
2. Sarcomas are characterized by cells that are located in bone, cartilage, fat, connective tissue, muscle, and other supportive tissues.
3. Lymphomas are cancers that begin in the lymph nodes and immune system tissues.
4. Leukemias are cancers that begin in the bone marrow and often accumulate in the bloodstream.
5. Adenomas are cancers that arise in the thyroid, the pituitary gland, the adrenal gland, and other glandular tissues.

Malignant cells are characterized by unrestrained growth, invasion of local tissues and potential metastasis to other tissues of the body (Murray, 1990). The process of Carcinogenesis, which is the transformation of normal cell into initiated cells subsequently leading to cancer cell, can be divided into three stages namely:

(1) Initiation: during which normal cells are initiated after exposure to a carcinogen.
(2) Promotion: a secondary effect following initiation that results in enhancement of cellular growth and division.
(3) Progression: during which already initiated cells continues to develop.
(4) Metastasis: during which malignant cells spread from initial site to invade other sites (Miaskowski and Buchsel, 1999).

1.2.2 MOLECULAR BASIS OF CARCINOGENESIS

The molecular basis of carcinogenesis is found in the defect or even absence of highly important genes that are involved in regulation of cell growth and apparently those involved in cell death that is growth promoting (proto-oncogenes), growth inhibiting (tumor suppressor genes), anti-oncogenes and genes controlling apoptosis (Bishop, 1995).

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- Oncogenes; these are derived from proto-oncogenes. They can either be viral oncogenes (v-onc) or cellular oncogenes (c-onc). Oncogenes encode protein products called oncoproteins which resemble the normal products of the proto-oncogenes, with the exception that oncoproteins are devoid of important regulatory elements and their production in the transformed cells does not depend on growth factors or other external signals (Weinberg, 1992). Oncogenes products include; growth factors (c-sis), growth factor receptors (v-erb B), signal-transducing protein (ras protein) and nuclear regulatory protein (c-myc) (Weinberg, 1992).
- Tumour suppressor gene; these are genes whose products apply 'brake' to cell proliferation. Mutation or deletion of these genes is like brake failure, permitting unrestrained cellular growth. Examples of these genes include; retinoblastoma (Rb) gene, p53 gene, adenomatous polyposis coli (APC) gene and necrosis factor-1 (NF-1) gene (Russo *et al*., 1998).
- Genes regulating apoptosis; Genes that prevent or reduce programmed cell death are also important in carcinogenesis. The prototypic gene in this category is bcl-2 and it is found to be over-expressed in certain cancers (Korsmeyer, 1992).

1.2.3 FACTORS INFLUENCING CARCINOGENESIS

Carcinogenesis is a protracted process that depends upon two main factors:

- Internal factors
- External factors.

1.2.3.1 INTERNAL FACTORS

These include immune function and genetic predisposition (Ames *et al*., 1995). Several immune agents such as macrohpages, natural killer cells, helper T lymphocytes, recognize some strange cell surface antigens on malignant cells and therefore destroy them, such that in the immunocompromised people cancer develop more freely (Cooper, 1995). Genetic predisposition is also noteworthy as certain factors like the location of specific proto-oncogenes within the genome is different and may provide an increased susceptibility to mutation or activation (Cooper, 1995).

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Also, the position of the oncogene may be normal but the genes controlling the oncogenes activity, the suppressor (or regulatory) gene may be abnormal or out of place (Calzone, 1997). BRCA1 and BRCA2 which are both ubiquitous cell cycle-regulated proteins that localize to the nucleus in normal cells. Heterozygous carriers of loss-of-function germline mutations in the *BRCA1* or *BRCA2* breast cancer susceptibility genes have a predisposition to breast and ovarian cancer (Vidarsson, *et al.*,2002). Persistently increased blood levels of estrogen are associated with an increased risk of breast cancer, as are increased levels of the androgens androstenedione and testosterone (which can be directly converted by aromatase to the estrogens estrone and estradiol, respectively). Increased blood levels of progesterone are associated with a decreased risk of breast cancer in premenopausal women. A number of circumstances which increase exposure to endogenous estrogens including not having children, delaying first childbirth, not breastfeeding, early menarche (the first menstrual period) and late menopause are suspected of increasing lifetime risk for developing breast cancer (Yager and Davidson, 2006).

1.2.3.2 EXTERNAL FACTORS

External (environmental) factors may be physical (radiation), biological (viral) or chemical (as with clastogens like arsenic). (Chai *et al*., 2003). Examples of environmental factors that have been associated with increased cancer risk in the human population include chemical and physical mutagens (e.g. cigarette smoke, heterocyclic amines, asbestos and UV irradiation), infection by certain viral or bacterial pathogens, and dietary non-genotoxic constituents (e.g. macro- and micronutrients). Among molecular targets of environmental influences on carcinogenesis are somatic mutation (genetic change) and aberrant DNA methylation (epigenetic change) at the genomic level and post-translational modifications at the protein level. At both levels, changes elicited affect either the stability or the activity of key regulatory proteins, including oncoproteins and tumor suppressor proteins. Together, via multiple genetic and epigenetic lesions, environmental factors modulate important changes in the pathway of cellular carcinogenesis.

A classic example of combined genetic and epigenetic changes is provided by the well-studied cellular response to UV irradiation, a potent etiologic agent in skin cancer

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development. While causing a signature mutation (Brash,1997), post-translational modifications triggered by UV irradiation have also been documented in the membrane, cytosol and nuclear compartments (Tommasi et al.,1997, Griffiths et al., 1998, Bender et al., 1997). In cellular plasma membrane, UV irradiation efficiently causes dimerization of receptors, as shown for IGFR and EGFR (Rosette. and Karin 1996, De-Metys et al., 1995). In the nuclei, UV irradiation causes DNA lesions that lead to the formation of pyrimidine dimers and subsequent signature mutations that coincide with activation of DNA-damage, related signaling cascades, as documented for c-*jun* N-terminal kinases (JNK) (Zanke e tal., 1996, Adler et al., 1995). Within the cytosol, UV irradiation has been implicated in the activation of various signaling cascades including protein kinase C (PKC) (Berra et al., 1997), mitogen activated protein kinase (MAPK) (Assefa et al., 1997), and JNK (Adler et al., 1996) with changes in downstream effectors, as shown for transcription factors cyclic AMP-response element binding protein (CREB) (Iordanov et al., 1997), p53 (Adler et al., 1997) and NFκB (Piette et al., 1997).

In general, dietary components relevant to cancer can be divided into three major categories: (i) dietary constituents that are carcinogenic including aflatoxins, heterocyclic amines, *N*-nitroso compounds, polycyclic aromatic hydrocarbons and trihalomethane (Nagao et al., 1997, Ames, 1983); (ii) dietary factors that promote tumor development (tumor promoters) including diverse chemical classes, such as phorbol ester derivatives, non-TPA type tumor promoters (Fujiki et al., 1989), chlorinated hydrocarbons (from industrial or agricultural sources), alcohol and salt (sodium chloride). Investigations of dietary effects on experimental tumor promotion (e.g. skin, breast, colon, liver) indicate that increased ingestion of fats and/or calories markedly enhances tumor promotion in most tissues examined (Birt et al., 1992, Nagao et al., 1997, Ames et al., 1983, Fujiki et al., 1989, Pariza et al., 1987).

1.2.4 MECHANISMS OF HEPATOCARCINOGENESIS

According to Raphael *et al.,*2006, hepatocellular carcinoma (HCC) is among the fifth most common cancers worldwide The causes of more than 85% of HCC cases are known (e.g. hepatitis B and C, aflatoxin B1, ethanol, metabolic diseases). HCC is an epithelial tumour developing from hepatocytes (Raphael *et al.,*2006). In the majority

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of cases, cirrhosis is the major underlying risk factor, but HCC may occur also on chronic hepatitis or normal liver. The development and progression of HCC are believed to be caused by the accumulation of genetic changes resulting in altered expression of cancer-related genes, such as oncogenes or tumour suppressor genes, as well as genes involved in different regulatory pathways. The genetic changes involved can be divided into four different pathways, each pathway contributing to a limited number of tumours. These are:

1. the p53 pathway involved in response to DNA damage,
2. the retinoblastoma pathway involved in control of the cell cycle,
3. the transforming growth factor-beta (TGF-beta) pathway involved in growth inhibition, and
4. the Wnt pathway involved in cell-cell adhesion and signal transduction (Raphael *et al.,*2006).

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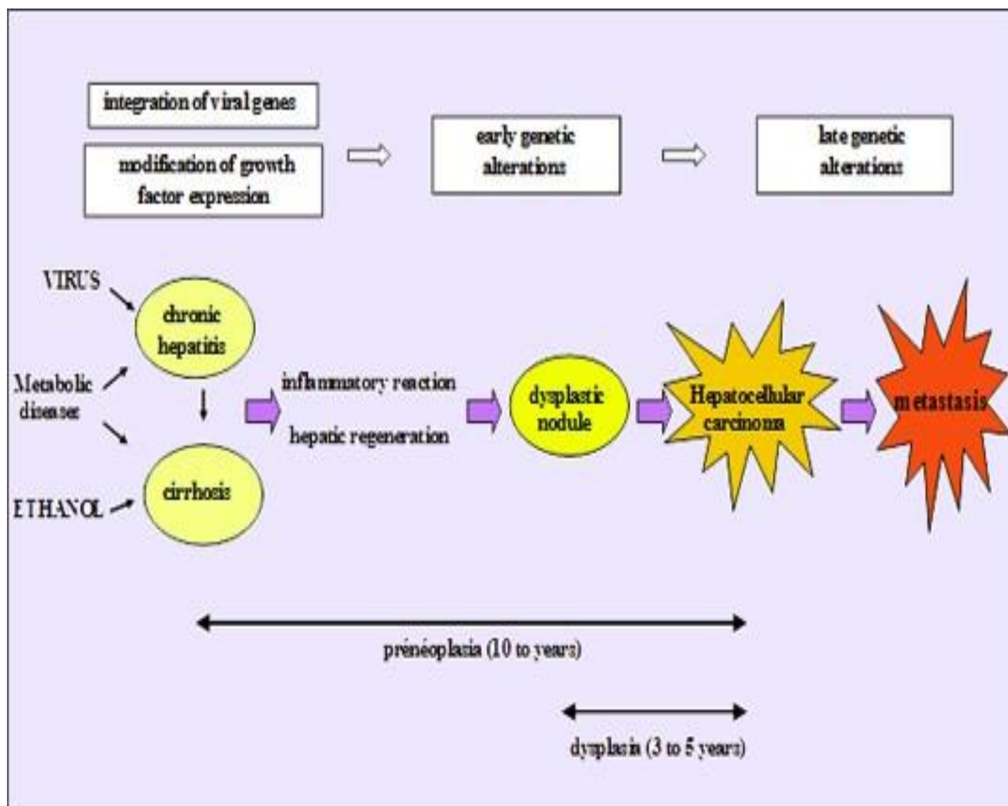

Figure 1.2: Sequential changes in the liver leading to HCC (Raphael *et al.*,2006).

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Hepatocarcinogenesis may begin in preneoplastic lesions such as macroregenerative nodules, low-grade and high grade dysplastic nodules (Takayama *et al.,*1990). Accelerated proliferation of hepatocytes and development of monoclonal hepatocyte populations occur in all preneoplastic conditions. Accumulation of genetic alterations in these preneoplastic lesions is believed to lead to the development of HCC (Wang *et al*., 2002). Genomic alterations occur randomly, and they accumulate in dysplastic hepatocytes and HCC. Although genetic changes may occur independently of etiologic conditions, some molecular mechanisms have been more frequently related to a specific etiology. For example, molecular pathways of HBV- and HCV-induced hepatocarcinogenesis involving Rb1, p53, and Wnt families are different from those associated with alcoholism (Wang *et al*., 2002).

1.3 CLASTOGENS AND CLASTOGENESIS

A clastogen is a material that can cause breaks in chromosomes, leading to sections of the chromosome being deleted, added, or rearranged. Therefore, Clastogenicity is described as the microscopically visible damages or changes to chromosomes (Testoni *et al*., 1997). This can cause a mutation , and lead to cancer development, as cells that are not killed by the clastogen may become cancerous. Known clastogens include acridine yellow, benzene, ethylene oxide, arsenic, phosphine and mimosine (Testoni *et al*., 1997).

Clastogens generally have low molecular weight (< 10,000 Daltons). Apart from causing chromosomal breakage, they can cause gene mutation, sister chromatid exchanges and other chromosomal aberrations. They were first described in the plasma of irradiated persons, but they are also found in hereditary breakage syndromes and chronic reactions (Testoni *et al*., 1997).

Some compounds have been identified to be carcinogenic, mutagenic as well as being clastogenic. For instance, Melq-2-amino-3,4-dimethylimidazo-(4,5-F) quinoline is a potent clastogen and carcinogen having been shown to develop fore-stomach and liver tumor (Ramsey *et al*., 1998). Also 2-acetylaminofluorene, besides acting as a mutagen has the capacity of causing chromosomal damage (Ames *et al*., 1972). Another potent and common clastogen is arsenic which may be present in water as contaminants.

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During cell division, the genetic material replicates and then divides equally between the two daughter cells that are produced. This process is disrupted by clastogens, which also cause chromosomal damage. When this occurs, the genetic material is not incorporated into a new nucleus, and then may form its own micronucleus which is clearly visible with a microscope (NTP, 2001). A positive correlation between the degree of clastogenicity and carcinogenicity has been established (Natarajan, 1984).

1.3.1 ARSENIC AND ITS COMPOUNDS

Arsenic is a naturally occurring element widely distributed in the earth's crust. It exists in three allotropic forms: yellow, black and grey; the stable form is a silver-grey, brittle crystalline solid. It tarnishes rapidly in air, and at high temperatures burns forming a white cloud of arsenic trioxide. Arsenic is a member of group V of the periodic table, which combines readily with many elements. The metallic form is brittle, tarnishes and when heated it rapidly oxidizes to arsenic trioxide, which has a garlic odour. The non metallic form is less reactive but will dissolve when heated with strong oxidizing acids and alkalis. Arsenic combines with other elements such as oxygen, chlorine, and sulphur to form inorganic arsenic compounds. Exposure to higher-than-average levels of arsenic occurs mainly in workplaces, near or in hazardous waste sites, and areas with high levels naturally occurring in soil, rocks, and water. Exposure to high levels of arsenic can cause death. Exposure to arsenic at low levels for extended periods of time can cause a discoloration of the skin and the appearance of small corns or warts.

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The word arsenic is derived from the Persian zarnikh, meaning ‘yellow orpiment’. It came to the Western languages through the Greek rendering of zarnikh: arsenikon, meaning ‘masculine’ in Greek. Arsenic sulphide also occurs in a red form: realgar or sandarach. Long known and used in Persia and elsewhere since ancient times, As was also used in traditional Chinese and Indian medicine and as a cometic product in eye shadow in the Roman era. Given that the symptoms of acute As poisoning are easily confused with diarrhea associated with cholera, it quickly became a favourite homicidal agent (Vahidnia, et al., 2007). Arsenic is a naturally occurring element that exists in the environment in a number of different forms, each with its own unique physical, chemical and toxicological characteristics. Arsenic naturally occurs in any of

its four valence states: -3 (arsine), 0 (elementary arsenic), +3 (arsenites), and +5 (arsenates). Elementary arsenic is a gray metallic-looking crystalline powder; arsine is a colorless gas; arsenites and arsenates are white crystalline powders. The term “arsenic” is used when the valence state is not specified and generally refers to arsenite and/or arsenate. The alkali salts are highly soluble in water but the calcium and lead salts are not. The pentavalent form, As^{5+} (arsenate or organic form) is less toxic than trivalent form, As^{3+} (arsenite or inorganic form) based on lower solubility. Both forms are found in arsenic-contaminated water, and they are inter-convertible once absorbed. Arsenic belongs to the group V elements of the periodic table and has atomic number of 33 and mass number of 76 (Chemistry word, 2008).

In chemistry, arsenite is a chemical compound containing an arsenic oxoanion where arsenic has oxidation state +3. Examples of arsenites include sodium arsenite which contains a polymeric linear anion, $[AsO_2^{-}]_n$, silver arsenite, Ag_3AsO_3, which contains the trigonal, AsO_3^{3-} anion, sometimes called ortho-arsenite (Greenwood *et al*., 1997). In fields that commonly deal with groundwater chemistry, arsenite are commonly referred to As_2O_3. Groundwater in several parts of the world contains substantial amounts of arsenic, primarily due to release of naturally occurring from subsurface rock formations (Nordstrom, 2002). Human exposure to naturally occurring arsenic in some world regions is significant because of the use of arsenic-contaminated groundwater as a primary source of drinking water (Chiou et al., 2001; Guo et al., 2004; NRC, 1999)

Arsenic compounds are acutely toxic, carcinogenic, teratogenic, and mutagenic. They are readily absorbed by various body tissues through the skin, respiratory and intestinal tracts, and transplacentally. They may cause severe irritation of tissues (skin, eyes, mucous membranes, and lungs). All arsenic compounds are moderately toxic (arsine is highly toxic), mutagenic in some but not all test systems, and teratogenic (ECRP, 1988).

Major uses of arsenic in various forms are as pesticides (insecticides, herbicides, and sheep and cattle dips) and in drugs. Sodium arsenite is used in the water gas shift reaction to remove carbon(IV)oxide.

1.3.1.1 FORMS AND OCCURRENCE

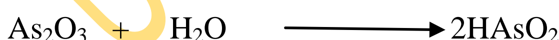
Arsenic compounds are naturally present everywhere and most metallic ores contain small quantities. Arsenical pyrites ($FeS_2.FeAs_2$), the sulphides realgar (As_2S_2) and Orpiment (As_2S_3) form the natural sources of arsenicals (Clarke and Clarke, 1975). Arsenic trioxide is normally produced in the roasting of metallic ores. This may be carried as a dust along with the smoke, and hence serve as a contaminant of soil, herbage and waste.

1.3.1.2 ARSENIC TRIOXIDE (As_2O_3)

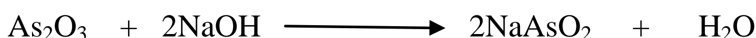
This is most common in general use. It occurs as an amorphous or crystalline, tasteless and odorless, white powder. It is sparingly soluble, volatile on heating, forming distinct glistering octahedral crystals on sublimation. It resembles flour and French chalk, which has led to several accidents. Copper arsenite, which was initially used as a cheap pigment for coloring wall-papers and artificial flowers, was stopped due to deaths that resulted from its use. However, it is being used as an insecticide antihelminthic and slug bait (Clarke and Clarke, 1975).

1.3.1.3 SODIUM ARSENITE ($NaAsO_2$)

Sodium arsenite is a trivalent inorganic compound belonging to general class of compound called arsenicals. It has molecular weight of 129.91 g/mol (Sigma, Research and Diagnostic Reagents Catalogue, 1994). It is an odourless and tasteless white-grey powder. It is toxic and the most effective agent of the arsenicals (Hodgson *et al*., 1988). Sodium arsenite is produced from arsenious acid (As_2O_3) by reacting with alkali such as arsenite and water (Lenihan, 1977).

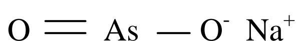


$$As_2O_3 + H_2O \longrightarrow 2HAsO_2$$



$$As_2O_3 + 2NaOH \longrightarrow 2NaAsO_2 + H_2O$$

Sodium arsenite is structurally represented as;



$$O = As - O^- \; Na^+$$

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1.3.1.4 ENVIRONMENTAL AND HEALTH EFFECTS OF ARSENIC

The arsenic cycle has broadened as a consequence of human interference and due to this, large amounts of arsenic end up in the environment and in living organisms. Arsenic is mainly emitted by the copper producing industries, but also during lead and zinc production and in agriculture. It cannot be destroyed once it has entered the environment. Therefore, the amounts thus added can spread and cause health effects to humans and animals on many locations on earth. Plants absorb arsenic fairly easily, so that high-ranking concentrations may be present in food. The concentrations of the dangerous inorganic arsenics that are currently present in surface waters enhance the chances of alteration of genetic materials of fish. This is mainly caused by accumulation of arsenic in the bodies of plant-eating freshwater organisms. Birds eat the fish that already contain eminent amounts of arsenic and will die as a result of arsenic poisoning as the fish is decomposed in their bodies.

Arsenic is one of the most toxic elements that can be found. Despite their toxic effect, inorganic arsenic bonds occur on earth naturally in small amounts. Humans may be exposed to arsenic through food, water and air. Exposure may also occur through skin contact with soil or water that contains arsenic. Levels of arsenic in food are fairly low, as it is not added due to its toxicity. But levels of arsenic in fish and seafood may be high, because fish absorb arsenic from the water they live in. Luckily this is mainly the fairly harmless organic form of arsenic, but fish that contain significant amounts of inorganic arsenic may be a danger to human health. Arsenic exposure may be higher for people that work with arsenic, for people that live in houses that contain conserved wood of any kind and for those who live on farmlands where arsenic-containing pesticides have been applied in the past. Exposure to inorganic arsenic can cause various health effects, such as irritation of the stomach and intestines, decreased production of red and white blood cells, skin changes and lung irritation. It is suggested that the uptake of significant amounts of inorganic arsenic can intensify the chances of cancer development, especially the chances of development of skin cancer, lung cancer, liver cancer and lymphatic cancer. A very high exposure to inorganic arsenic can cause infertility and miscarriages with women, and it can cause skin disturbances, declined resistance to infections, heart disruptions

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and brain damage with both men and women. Finally, inorganic arsenic can damage DNA.

Arsenic compounds are widely distributed in the biosphere and on the earth's crust (Lenihan and Fletcher, 1977). Arsenic is a common environmental toxicant found in soil, water and air. Significant exposure to arsenic occurs via both anthropogenic and natural sources. Arsenic is released into the air by volcanoes and is a natural contaminant of some deep water wells. Occupational exposure to arsenic is common in the smelting industry (in which arsenic is a byproduct of ores containing lead, gold, Zinc, cobalt, and nickel) and is increasing in the microelectronics industry (in which gallium arsenite is responsible). Low level arsenic exposure continues to take place in the general population (as do some cases of high dose poisoning) through the commercial use of inorganic arsenic compounds in common products, such as wood preservatives, ant-killers, herbicide, fungicides, through the consumption of foods and smoking of tobacco treated with arsenic-containing pesticides and through burning of fossil fuels in which arsenic is a contaminant (HUH, 2001).

1.3.1.5 USES OF SODIUM ARSENITE

Sodium arsenite is widely used as weed killer (Herbicides), dressing of grain, insect poisons (insecticides), cattle and sheep-dips and wood preservatives and debarking of trees. Reynolds (1999) reported that over 8,000 arsenic based compounds were used to treat asthma, malaria, tuberculosis, diabetes and skin diseases and sleeping sickness.

The agricultural industry utilizes arsenic in the form of monosodium methylarsenite (MSMA), disodium methyl arsenite (DSMA), cacodylic acid (dimethyl arsenic acid) and arsenic acid (Othmer, 1986). They are used for the control of Johnson and nusedge grass and weeds in cotton fields as herbicide application. The use of inorganic arsenicals (sodium arsenite and arsenic trioxide) as herbicides has been reduced greatly because of livestock losses, environmental persistence, and their association with carcinogenesis. Arsenic derivatives continue to be available in other parts of the world in wood preservatives and insecticide formulations. These compounds can be hazardous to animals when used as recommended. Ruminants (even deer) are apparently attracted to and lick plants poisoned with arsenite. The highly soluble

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organic arsenicals (methane arsonate, methyl arsonic acid) can concentrate in pools in toxic quantities after a rain has washed them from recently treated plants. Arsenicals are used as desiccants or defoliants on cotton, and residues of cotton harvest fed to cattle may contain toxic amounts of arsenic.

1.3.1.6 DISTRIBUTION

Sodium arsenite is distributed to other tissues from the liver where it accumulates and excess can be stored in bone, skin, and keratinized tissues such as hair and hooves. Arsenic initially localizes in the blood bound to globulin. Redistribution occurs within 24 hours to the liver, lungs, intestinal wall, and spleen, where arsenic binds to sulfhydryl groups of tissue proteins only small amounts of arsenic penetrates the blood-brain barrier (Winsk *et al*., 1995). There is also a significant accumulation of arsenate in the skeleton, presumably by exchange with phosphate (Lindgren *et al*., 1983). Application of pentavalent arsenic to skin results first in an accumulation of arsenic in the skin, followed by distribution to other organs, followed by urinary excretion. Significant deposition in hair and nails has been demonstrated in man and animals.

1.3.1.7 ARSENIC METABOLISM

Arsenic absorption occurs mainly in the small intestines. A minimal absorption also takes place from skin contact and inhalation. The rate of absorption of inorganic arsenicals from digestive tract depends upon their solubility. Sodium arsenite is readily soluble, rapidly absorbed and highly toxic (Calesnick *et al*., 1966). The soluble arsenic compounds get easily absorbed by contact with intact skin and absorption from a fresh wound is very rapid.

Accumulation of arsenic is seen in the liver with slow release and distribution to other tissues. Continued administration can cause its disappearance from soft tissues and its long term storage in bones, skin and keratinized tissues (for example, hair and hoof) (Grollman and Slaughter, 1947). Arsenic deposited in hair is irremovable and moves slowly along the hair as it grows. Arsenic is excreted in the urine, faeces, sweat and milk. The rate of excretion depends on the compound and it is inversely related to the toxicity (Sabeh *et al*., 1993).

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Pentavalent arsenic is well absorbed through the gut, but the trivalent form is more lipids soluble. Toxicity results from the arsenite form (As^{3+}), especially by dermal absorption. Arsenic compounds are well absorbed parenterally within 24 hours. Sodium arsenite is readily soluble, rapidly absorbed and highly toxic (Sabeh *et al*., 1993).

Arsenites and arsenates are absorbed by ingestion and parenteral injection; sodium arsenite, arsenic trioxide, and arsine are absorbed by inhalation. Sodium arsenite and arsenate are absorbed through the placenta.
Arsenic is biotransformed in vivo by methylation to monoethyl and dimethyl arsenic. Dimethylarsenic is the principal transformation product and it appears to be the terminal metabolite which is rapidly formed. Dimethylarsenic promotes lung and skin tumor by way of the metabolic production of free radicals such as dimethyl arsenic peroxyl radical [$(CH_3)_2AsO$]. Dimethylarsenic may play an important role in arsenic carcinogenesis through induction of oxidative damage, particularly of base oxidation especially in the target organs of arsenic carcinogenesis; skin, lung, liver, and urinary bladder (Jayanthika *et al*., 2001). Sodium arsenite produces alteration in the RNA and DNA synthesis in cells, this inhibition is said to be dose-dependent (Goering *et al*; 1999).

The possible mechanism of this cytotoxic effect of arsenite might be due to its reactivity with intracellular Sulfhydryl groups.

The biotransformation of arsenic is based on several generally accepted steps. For methylation to occur arsenate (As^{5+}) species must be reduced to arsenite (As^{3+}) in a process that occurs through reactions involving glutathione (GSH). The arsenite is then methylated to monomethlarsonic acid (MMA). The MMA is then methylated to dimethylarsinic acid (DMA) (Goering *et al*; 1999).

Oxidative addition of methyl groups to arsenic occurs by methyltransferase enzymes, with S-adenosyl-methionin (SAM) as the methyl-donating cofactor (Aposhian *et al*., 1997 and Thompson, 1993).

In addition to the pentavalent metabolites, the trivalent metabolites, monomethylarsonous acid [MMA (111)] and dimethylarsinous acid [DMA (111)], have been identified as intermediary metabolites in the methylation of arsenic compounds and have been detected in

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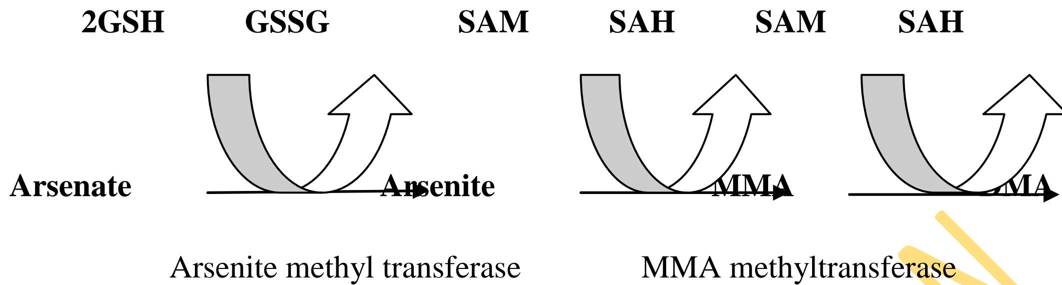
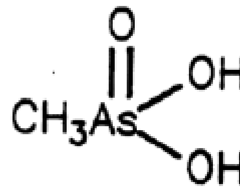
cultured human cells treated with inorganic arsenic (Thomas *et al*., 2001). Mandal *et al* reported the presence of MMA(III) and DMA(III) in the urine of people chronically exposed to inorganic arsenic via drinking water in West Bengal, India (Mandal *et al.,* 2001).



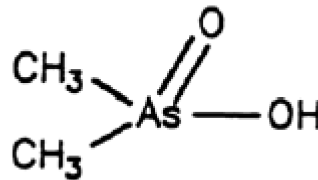
The metabolic pathway for arsenic (Goering *et al*., 1999).

1.3.1.8 PRODUCTS OF ARSENIC METABOLISM

Monomethylarsonic Acid (MMA)



Dimethylarsonic Acid (Cacodylic acid, DMA)



Structure of Arsenic metabolic products.

1.3.1.9 ARSENIC TOXICITY

Arsenic toxicity (poisoning) is a medical condition caused by the elevated levels of arsenic in the body. The dominant basis of arsenic poisoning is from ground water that naturally contains high concentrations of arsenic. A 2007 study found that over 137 million people in more than 70 countries are probably affected by arsenic poisoning of drinking water (Smedley et al., 2002). Symptoms of arsenic poisoning begin with headaches, confusion, severe diarrhoea, and drowsiness. As the poisoning develops, convulsions and changes in fingernail pigmentation called leukonychia may occur.

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When the poisoning becomes acute, symptoms may include diarrhoea, vomiting, blood in the urine, cramping muscles, hair loss, stomach pain, and more convulsions. The organs of the body that are usually affected by arsenic poisoning are the lungs, skin, kidneys, and liver. The final result of arsenic poisoning is coma to death. Arsenic is related to heart disease (Tseng et al., 2003), (hypertension related cardiovascular), cancer, (Smith et al., 1992) stroke (Chiou et al., 1997) (cerebrovascular diseases), chronic lower respiratory diseases, (Hendryx et al., 2009) and diabetes (Navas-Acien et al., 2008, Kile et al., 2008).

Long term exposure to arsenic is related to vitamin A deficiency which is related to heart disease and night blindness (Hsueh et al., 1998).

Research has shown that the inorganic arsenites (trivalent forms) in drinking water have a much higher acute toxicity than organic arsenates (pentavalent forms) (Kingston et al., 1993).The acute minimal lethal dose of arsenic in adults is estimated to be 70 to 200 mg or 1 mg/kg/day (Dart, 2004). Most reported arsenic poisonings are caused by one of arsenic's compounds, also found in drinking water, arsenic trioxide which is 500 times more toxic than pure arsenic.

In general, arsenites are much more toxic than arsenates; the oral LD_{50} of arsenates in rats and mice is about 100 mg/kg and that of arsenites about 10 mg/kg; the acute oral LD_{50} of arsenic trioxide is 15 mg/kg in rats and 39 mg/kg in mice. Although rare, acute poisoning is reported in children (Gullen *et al.,* 1995). Acute and chronic effects of arsenic intoxication in man have been summarized (IARC, 1980). They include a burning sensation of mouth and throat; metallic, garlicky odor of breath and feces; difficulty in swallowing; vomiting; diarrhea; and cyanosis. Chronic effects include hyperpigmentation and keratosis (characteristics of prolonged treatment with Fowler's solution), vascular effects ("blackfoot disease"), cirrhosis of the liver, and effects on the hematopoietic system (leukopenia, anemia). The chief toxic effect of inhaled arsine is due to its binding to hemoglobin, resulting in extensive hemolysis and hematuria followed by jaundice; the usual cause of death is renal failure (Gullen *et al.,* 1995).

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1.3.2.1 BASIS OF ARSENITE-INDUCED TOXICITY

Arsenite interacts with thiol-containing amino acids, peptides and proteins (Winsk *et al*., 1995). Arsenite exerts its cellular toxicity by binding to sulflhydryl groups which results in enzyme inhibition.

During arsenic metabolism, oxygen radical may be produced, possibly leading to damage of DNA, proteins, lipids and other molecules. There is a positive correlation between lipid peroxidation and arsenic tissue concentrations in the livers, kidneys and heart of arsenite treated rats (Rasmos *et al.*, 1995). Arsenite induces the body's antioxidant activities in human firoblasts (Lee *et al*., 1995). It induces heme oxygenase, leading to the heme degradation iron release and decrease in the cytochrome p450 biotransformation enzymes important in both endogenous and xenobiotic metabolism (Albores *et al.,* 1989). Because of arsenite affinity for protein sulfhydryls, many side effects can occur from enzyme inhibition. Chronic arsenite toxicity results in mitochondrial changes that block lipoic acid-dependent dehydrogenase, which in turn inhibits glycolysis and results in demand for glucose and subsequently hypoglycemia (Cobo *et al*., 1995). Arsenicals also inhibit pyruvate dehydrogenase in gluconeogenesis (Szinicz'L *et al*., 1988). Carbohydrate depletion caused by gluconeogenesis depletion may therefore aggravate arsenic toxicity.

Increasing evidence indicates that arsenic acts on signaling pathways that regulate cell proliferation rather than causing direct DNA damage because arsenic exhibits its mutagenic activities only at concentrations high enough to also produce cell damage (Jacobson-Kram and Montalbano, 1985). Arsenic has been shown to modulate the mitogen-activated protein AP-1 (Cavigelli *et al*., 1996). AP-1 mediates many biological effects of tumor promoters and is an important regulator of cell growth. The ability of arsenic to interact with protein thiol groups on key regulatory proteins and subsequently alters their activities is likely to contribute to this effects (Cavigelli *et al*., 1996). Accordingly, it has been demonstrated that arsenic can induce a moderate increase in keratinocyte cell proliferation, as evidenced by increases in thymidine incorporation (Germolec *et al*., 1998), cell cycling (Klimecki *et al*., 1996), labeling of the proliferating cell marker Ki-67 (Klimecki *et al*., 1996), ornithine decarboxylase

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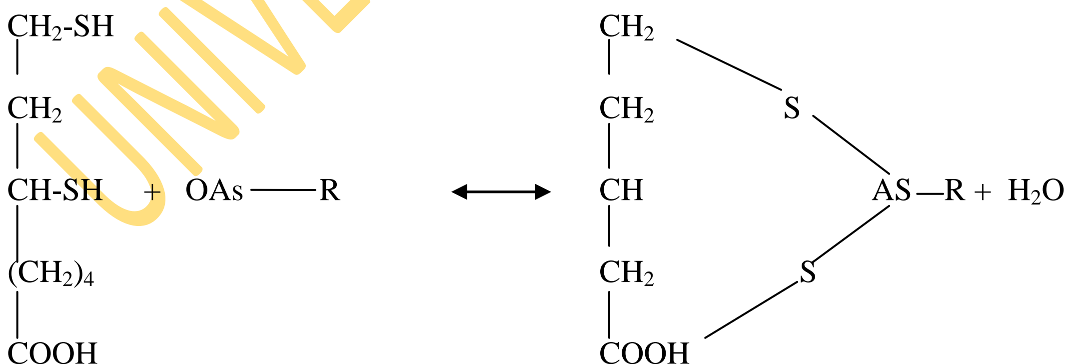
activity (Brown and Kitchin, 1996), and expression of oncogenes and growth factors such as c-*fos,* c-*jun,* c-*myc,* and transforming growth factor (Germolec *et al*., 1998).

1.3.2.2 CARCINOGENIC EFFECTS

The IARC classify arsenic as a carcinogen for which there is sufficient evidence from epidemiological studies to support a causal association between exposure and skin cancer. Chronic arsenic exposure has also been associated with a greatly elevated risk of skin cancer and possibly of cancer of the lung, liver, angiosarcoma, bladder, kidney and colon cancers (ECRP, 1988).They also detected elevation of serum globulins and development of esophaged varices at follow up studies. There is also some evidence suggesting of changes in choloestatic function of the liver as shown by conjugated hyperbilirubinemia and elevated alkaline phosphatase activity, which directly relates to the concentration of total arsenic in urine (Ellenhern, 1997).

Pulmonary findings of chronic arsenic toxicity include both obstructive and restrictive patterns of pulmonary function tests (Ellenhern, 1997).Arsenicals have been shown to cause enzymatic inhibition of the tricarboxylic acid (TCA) cycle (Webb, 1996). Pyruvate dehydrogenase system has been shown to be especially sensitive to trivalent arsenicals because of their apparent interaction with disulphdryl lipoic acid moiety of this system as shown below:



Enzymatic inhibition reactions of Sodium Arsenite (Webb, 1966).

Sodium arsenite was found to inhibit methyl thymidine uptake in human cells *in-vitro*, consistent suppression of DNA synthesis. Chromosomal aberrations were observed in

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human leucocyte exposed to sodium arsenite. Arsenic has also been suggested to substitute for phosphorus in DNA, causing a weak bond in DNA chain (Petres *et al.*, 1970). The toxicity of trivalent arsenic to animals and human beings has also been thought to be caused by its binding to thiol ions, thus inhibiting enzymatic reactions. As late as 1980 it was believed that arsenic compounds were not carcinogenic in experimental animals, and this conclusion was drawn from a summary of largely negative results (IARC, 1980). Since that time evidence has appeared which indicates carcinogenicity in rats (Ivankovic *et al.*, 1979). The evidence for carcinogenicity of arsenic compounds in man is more positive, and this has been reviewed (Landrigan, 1981; IARC, 1980). A correlation was established between the appearance of skin cancer and arsenic concentration in the well water in certain regions of Taiwan (Tseng, 1977). Skin cancers were also noted repeatedly in patients after prolonged treatment with Fowler's solution (potassium arsenite) and in vineyard workers employing arsenical pesticides. Lung cancers have been noted in men involved in the production of arsenicals (Mabuchi *et al.*, 1980). Other studies (involving workers in copper smelters and mines) are not as Clear-out since exposure to other materials occurred concomitantly.

1.3.2.3 USES OF ARSENIC

Arsenic compounds are used in making special types of glass, as a wood preservative and, lately, in the semiconductor gallium arsenade, which has the ability to convert electric current to laser light. Arsine gas AsH_3, has become an important dopant gas in the microchip industry, although it requires strict guidelines regarding its use because it is extremely toxic. The main use of metallic arsenic is for alloying with copper and especially lead. Lead components in car batteries are strengthened by the presence of a few percent of arsenic. Gallium arsenide (GaAs) is an important semiconductor material, used in integrated circuits. Circuits made from GaAs are much faster (but also much more expensive) than those made in silicon. Unlike silicon it is direct bandgap, and so can be used in laser diodes and LEDs to directly convert electricity into light (Sabina et al., 2005).

Some species of bacteria obtain their energy by oxidizing various fuels while reducing arsenates to form arsenites using reductases enzymes. In 2008, bacteria were

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discovered that employ a version of photosynthesis with arsenites as electron donors, producing arsenates (just like ordinary photosynthesis uses water as electron donor, producing molecular oxygen). The researchers conjectured that historically these photosynthesizing organisms produced the arsenates that allowed the arsenate-reducing bacteria to thrive. In humans, arsenite inhibits Pyruvate Dehydrogenase (PDH complex) in the acetyl CoA reaction and binds to the thiol group of Lipoamide, a pyruvate participant coenzyme. In this inhibition, arsenite poisoning affects energy production in the body.

1.4. ETHANOL (ALCOHOL) AND HEPATOTOXICITY

Ethanol, a type of alcohol, is a colourless liquid with pleasant smell and a molecular formula C_2H_5OH.

Alcohol-related liver diseases are complex, and ethanol has been shown to interact with a large number of molecular targets. Ethanol can interfere with hepatic lipid metabolism in a number of ways and is known to induce both inflammation and necrosis in the liver. Ethanol increases the formation of superoxide by Kupffer cells thus implicating oxidative stress in ethanol-induced liver disease (Ernest, 2004). Similarly prooxidants (reactive oxygen species) are produced in the hepatocytes by partial reactions in the action of CYP2E1, an ethanol-induced CYP isoform. The formation of protein adducts in the microtubules by acetaldehyde, the metabolic product formed from ethanol by alcohol dehydrogenase, plays a role in the impairment of VLDL secretion associated with ethanol (Ernest, 2004).

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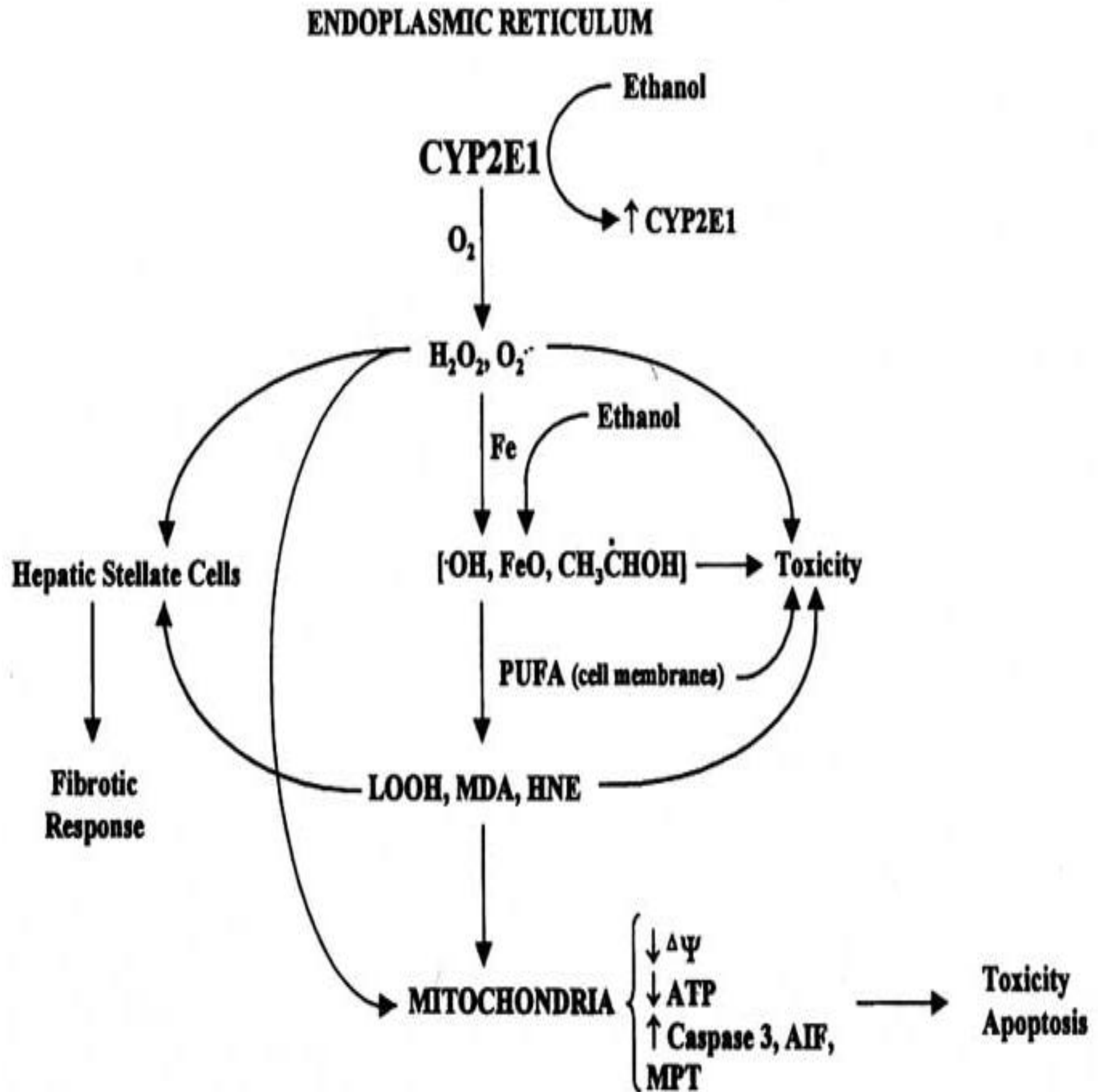

Figure 1.3: CYP2E1 Mediated Ethanol – Induced Hepatotoxicity

Source: (Cederbaum, 2006)

1.4.1 ETHANOL, OXIDATIVE STRESS AND FREE RADICAL DAMAGE

Reactive oxygen species (ROS) are small, highly reactive, oxygen-containing molecules that are naturally generated in small amounts during the body's metabolic reactions and can react with and damage complex cellular molecules such as fats, proteins, or DNA. Alcohol (ethanol) promotes the generation of ROS and/or interferes with the body's normal defense mechanisms against these compounds through numerous processes, particularly in the liver. For example, alcohol breakdown in the liver results in the formation of molecules whose further metabolism in the cell leads to ROS production. Alcohol also stimulates the activity of enzymes called cytochrome P450s, which contribute to ROS production. Further, alcohol can alter the levels of certain metals in the body, thereby facilitating ROS production. Finally, alcohol reduces the levels of agents that can eliminate ROS (i.e., antioxidants). The resulting state of the cell, known as oxidative stress, can lead to cell injury. ROS production and oxidative stress in liver cells play a central role in the development of alcoholic liver disease.

Oxidative stress represents an imbalance between the production and manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Some reactive oxidative species can even act as messengers in redox signaling.

In humans, oxidative stress is thought to be involved in the development of many diseases or may exacerbate their symptoms. These include cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, Schizophrenia, Bipolar disorder, fragile X syndrome, Sickle Cell Disease, and chronic fatigue syndrome (De Diego-Otero,et al., 2009). However, reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens. Short-term oxidative stress may also be important in prevention of aging by induction of a process named mitohormesis.

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Free radicals are molecules or fragments of molecules containing unpaired electron in their outermost orbitals (José, 2003). A free radical is an atom, molecule, or compound that is highly unstable because of its atomic or molecular structure (i.e., the distribution of electrons within the molecule). As a result, free radicals are very reactive as they attempt to pair up with other molecules, atoms, or even individual electrons to create a stable compound. To achieve a more stable state, free radicals can "steal" a hydrogen atom from another molecule, bind to another molecule, or interact in various ways with other free radicals.

The involvement of free radical mechanisms in the pathogenesis of alcoholic liver disease (ALD) is demonstrated by the detection of lipid peroxidation markers in the liver and the serum of patients with alcoholism, as well as by experiments in alcohol-feed rodents that show a relationship between alcohol-induced oxidative stress and the development of liver pathology. Ethanol-induced oxidative stress is the result of the combined impairment of antioxidant defences and the production of reactive oxygen species by the mitochondrial electron transport chain, the alcohol-inducible cytochrome P450 (CYP) 2E1 and activated phagocytes (Emanuele, 2006).
Furthermore, hydroxyethyl free radicals (HER) are also generated during ethanol metabolism by CYP2E1. The mechanisms by which oxidative stress contributes to alcohol toxicity are still not completely understood. The available evidence indicates that, by favouring mitochondrial permeability transition, oxidative stress promotes hepatocyte necrosis and/or apoptosis and is implicated in the alcohol-induced sensitization of hepatocytes to the pro-apoptotic action of TNF-a. Moreover, oxidative mechanisms can contribute to liver fibrosis, by triggering the release of pro-fibrotic cytokines and collagen gene expression in hepatic stellate cells. However, the reactions of HER and lipid peroxidation products with hepatic proteins stimulate both humoral and cellular immune reactions and favour the breaking of self-tolerance during ALD. Thus, immune responses might represent the mechanism by which alcohol-induced oxidative stress contributes to the perpetuation of chronic hepatic inflammation (Emanuele, 2006).

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1.4.2 ANTIOXIDANTS

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Halliwell and Gutteridge (1999) defined an antioxidant as 'any substance, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate'. This definition includes compounds of a non-enzymatic as well as an enzymatic nature. Clearly, the diversity of antioxidants matches that of pro-oxidants (Sies, 1993). Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Sies, 1997).

An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed 'oxidative stress'. Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions. Antioxidant defence involves several strategies, both enzymatic and non enzymatic. In the lipid phase, tocopherols and carotenes as well as oxy-carotenoids are of' interest, as are vitamin A and ubiquinols. In the aqueous phase, there are ascorbate, glutathione and other compounds. In addition to the cytosol, the nuclear and mitochondrial matrices and extracellular fluids are protected. Overall, these low molecular mass antioxidant molecules .add significantly to the defense provided by the enzymes superoxide dismutase, catalase and glutathione peroxidases (Sies, 1997).

Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials with a limited number of antioxidants detect

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no benefit and even suggested that excess supplementation with certain putative antioxidants may be harmful.

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1.4.2.1 ANTIOXIDANTS AND ALCOHOLIC LIVER DISEASE

Reactive oxygen species (ROS) act as signalling intermediates regulating multiple cellular processes. The fate and disposal of the signalling species are determined by the actions of antioxidants, particularly glutathione (GSH). The mitochondrial pool of GSH (mGSH) arises from the transport of cytosol GSH by a specific mitochondrial carrier and is responsible for the maintenance of a healthy competent organelle. The depletion of mGSH upon impairment of the mitochondrial transport activity leaves mitochondria unprotected from damaging effects of ROS overgeneration within the mitochondrial electron transport chain. Tumor necrosis factor-α (TNF-α) has emerged as a key player in the progression of the alcohol-induced liver disease (ALD), and is known to target mitochondria.

Key components of TNF signaling include sphingolipids, particularly ceramide generated from acidic sphingomyelinase activation serving as a source for gangliosides. In experimental models alcohol consumption enhances cholesterol levels and subsequent deposition into mitochondria resulting in selective decrease in the mGSH stores which is sufficient by itself to sensitize hepatocytes to TNF-α-mediated cell-death. Thus, the combination of TNF-α overproduction, enhanced glycosphingolipid generation and selective mGSH depletion by alcohol intake cooperate making the liver sensitive to alcohol.

Ethanol-induced oxidative stress is as a result of the combined impairment of antioxidant defences and the production of reactive oxygen species by the mitochondrial electron transport chain, the alcohol-inducible cytochrome P450 (CYP) 2E1 and activated phagocytes (Aparajita and Cederbaum, 2006). Furthermore, hydroxyethyl free radicals (HER) are also generated during ethanol metabolism by CYP2E1. The available evidence indicates that, by favouring mitochondrial permeability transition, oxidative stress promotes hepatocyte necrosis and/or apoptosis and is implicated in the alcohol-induced sensitization of hepatocytes to the pro-apoptotic action of TNF-a. Moreover, oxidative mechanisms can contribute to liver fibrosis, by triggering the release of pro-fibrotic cytokines and collagen gene expression in hepatic stellate cells (Emanuele, 2006).

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1.5 AFRAMOMUM LONGISCAPUM

1.5.1 CLASSIFICATION

Kingdom:Plantae

(unranked):Angiosperms

(unranked):Monocots

(unranked):Commelinids

Order:Zingiberales

Family:Zingiberaceae

Genus:*Aframomum*

Species: *A. longiscapum*

Yoruba: Ataare

Ibo: Upa

Aframomum Longiscapum is a species in the ginger family, Zingiberaceae. This is a family of flowering plants consisting of aromatic perennial herbs with creeping horizontal or tuberous rhizomes, comprising 52 genera and more than 1300 species, distributed throughout tropical Africa, Asia, and the Americas (Zomlefer, 1994). The Zingiberaceae constitute a family of terrestrial rhizomal herbs with over 1400 species distributed in over 50 genera (Hepper, 1996). They are mostly found in tropical areas like Asia and Africa (Koechlin,1965). In West and Central Africa plants of the Zingiberaceae family are widespread in humid forest regions. They are distributed among eight genera five of which are indigenous or endemic (*Aulotandra, Costus, Kampferia, Reneilmia* and *Aframomum*). The three others (*Pheaeomeri, Zingiber* and *Curcuma*) have been introduced. Many species are widely used for medicinal, ethnodietary and spiritual purposes, prompting chemical investigation. The classes of compounds generally found in *Aframomum* species include diterpenoids, sesquitepenoids, arylalkanoids and flavonoids. Some extracts and compounds from this genus have been screened for biological activities. These include antifungal, cytotoxic, antibacterial, antiplasmodial, antihypercholesterolemic and antiviral activities (Tane *et al.,*2002). However, many species are important ornamental plants, spices, or medicinal plants. Members of the family are small to large herbaceous plants

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with distichous leaves and basal sheaths that overlap to form a pseudostem. The plants are either self-supporting or epiphytic. Flowers are hermaphroditic, usually strongly zygomorphic, in determinate cymose inflorescences, and subtended by conspicuous, spirally arranged bracts. The perianth is composed of two whorls, a fused tubular calyx, and a tubular corolla with one lobe larger than the other two. Flowers typically have two of their stamenoids (sterile stamens) fused to form a petaloid lip, and have only one fertile stamen. The ovary is inferior and topped by two nectaries, the stigma is funnel-shaped (Tane *et al.,*2002). *Aframomum Longiscapum* gives a pungent, peppery flavour. Although it is native to West Africa, it is an important cash crop in the Basketo special woreda of southern Ethiopia. The pungent, peppery taste of the seeds is caused by aromatic ketones such as 1-(4-hydroxy-3-methoxyphenyl)-decan-3-one).

In Asia, zingiberaceous plants have been used since ancient times as both spices and medicines, such as in traditional Chinese medicine. The rhizomes of these plants are usually aromatic, and are used to treat indigestion, hepatitis, jaundice, diabetes, atherosclerosis and bacterial infections (Kuhn et al., 2001 and Van Wyk et al., 2004).

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Figure 1.4: *Aframomum longiscapum* plants.

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Figure 1.5: Ripe fruits of *Aframomum longiscapum* still attached to the plants.

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Figure 1.6: *Aframomum longiscapum* fruits

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Figure 1.7: *Aframomum longiscapum* grains

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1.5.2 PHYTOCHEMICAL CONTITUENTS OF AFRAMOMUM SPECIES

The *Aframomum* species as well as other Zingiberaceae are best known for the production of labdane diterpenoids and flavonoids. Other classes of compounds encountered in the genus include sesquiterpenoids and arylalkanoids. Out of the species that have been chemically studied, at least eleven contain diterpenoids. The phytochemcial analysis carried out on *Aframomum longiscapum* revealed the presence of alkaloids, saponin, flavonoid and cardenol. The presence of these phytochemcials support the use of this plant as antimicrobial agent.

1.5.2.1 Diterpenoids

Aframodial (**1**), aulacocarpinolide (**2**), aulacocarpin A (**3**) and aulacocarpin B (**4**) have been isolated from *A. aulacocarpos* (Ayafor *et al.,*1994; Ayafor *et al.,*1994). Aframodial (**1**) was isolated for the first time from *A. danielli* (Kimbu *et al.,*1979). It has also been obtained from *A. polyanthum, A. masuianum, A. keyserianum*(5g/kg dry weight of seeds)*, A. sulcatum,*(Tsopmo *et al.,*2002) *A. longifolius, (Tatsimo et al.,* 2005), *A. arundinaceum* (Wabo and Tane, n.d), and *A. latifolium*. Compounds **3** and **4** were also found in the seeds of *A. escapum.*(Ayimle *et al.,* 2004).

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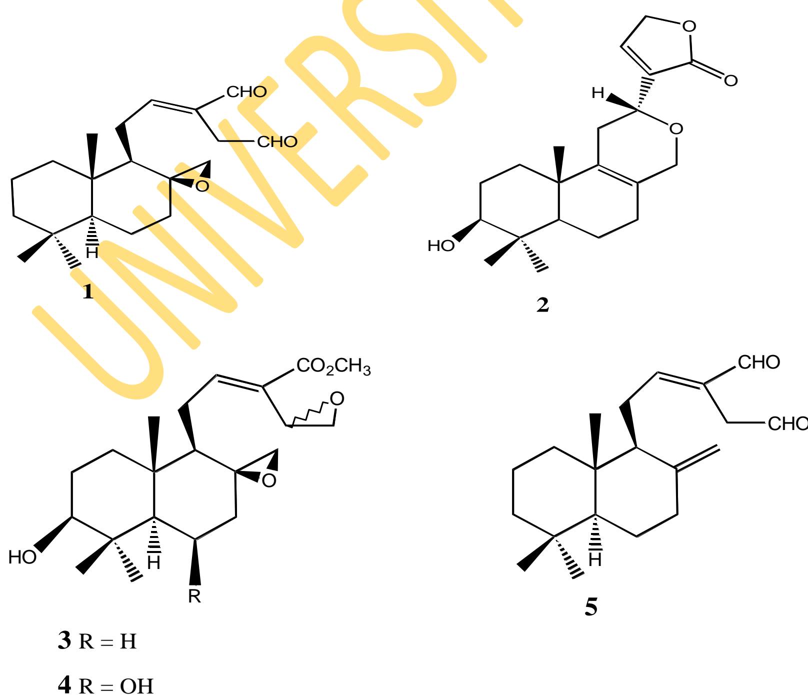
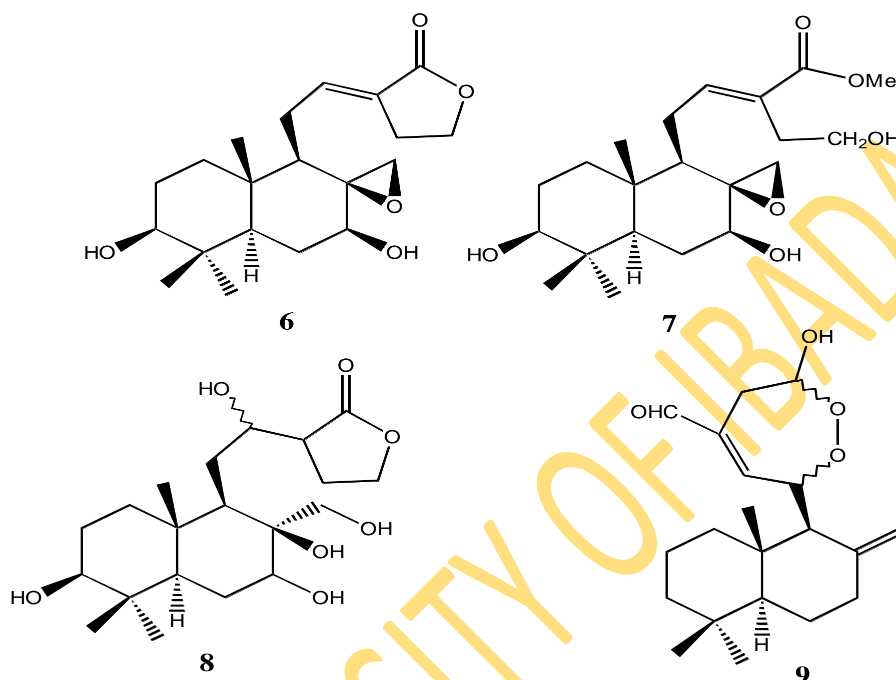


Structures of diterpenoids

Labda-8(17),12-dien-15,16-dial (**5**) has been found in *A. sceptrum*,(Duker-Eshun *et al.*,2002), *A. longifolius* (Morita and Itokawa, 1998), and *A. danielli*. (Kimbu *et al.*,1987). Many other diterpenoids were isolated from *A. sceptrum*. These include 8β(17)-epoxy-3 β,7 β -dihydroxylabd-12(E)-en-16,15-olide (**6**), methyl 8 β(17)-epoxy-3 β,7 β,15-trihydroxylabd-12(E)-en-16oate(**7**),3 β,7 β,8 β,12ζ,17pentahydroxylabdan-16,15-olide (**8**), coronarin B (**9**). ,(Duker-Eshun *et al.*,2002).



Stuctures of diterpenoids

Galanolactone (**10**), galanal A (**11**) and B (**12**) were obtained from *A. sulcatum* and *A. latifolium*,(Tsopmo *et al.*,2002), Other diterpenoids with 8(17)-epoxy moiety were isolated from *A. sulcatum*. These include a norbislabdane sulcanal (13), 12(E), 8β(17)-epoxy-11-hydroxy-12-labden-15,16-dial-11,15-hemiacetal **14** and **15**.,(Tsopmo *et al.*,2002).

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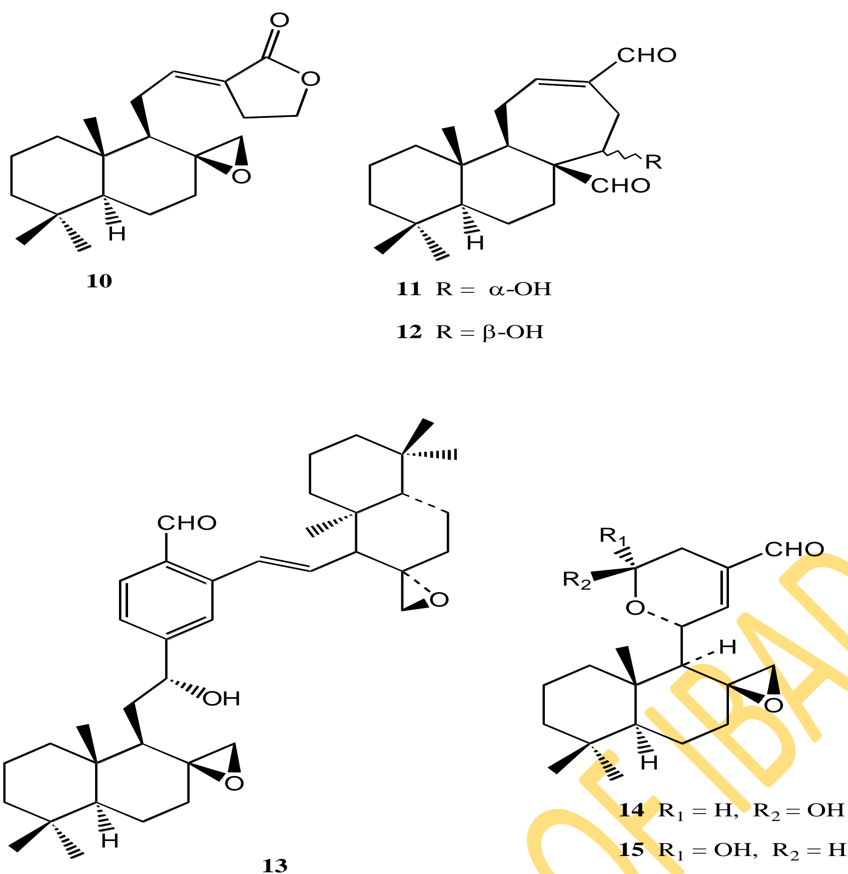
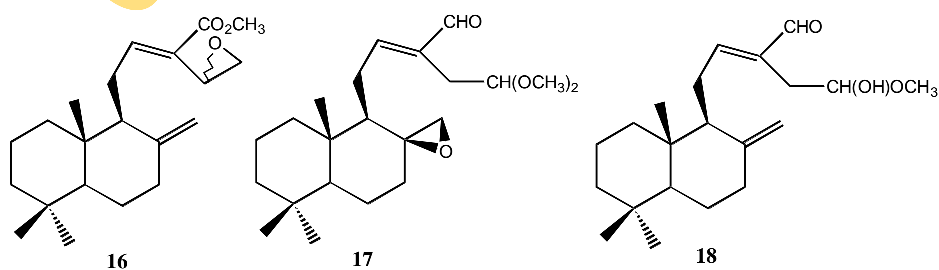


Stuctures of diterpenoids

Further studies on *A. danielli* (Kimbu *et al.,*1987). and *A. arundinaceum* (Wabo and Tane, n.d), yielded methyl 14,15-epoxy-8(17),12(E)-labdadiene-16-oate (**16**). An acetal, 8β(17)-epoxy-15,15-dimethoxylabd-12(E)-en-16-al (**17**) and an hemiacetal 15-hydroxy-15-methoxylabda-8(17),12(E)-dien-16-al (**18**) were isolated from *A. longifolius*. (Tatsimo *et al.,* 2005).



Stuctures of diterpenoids

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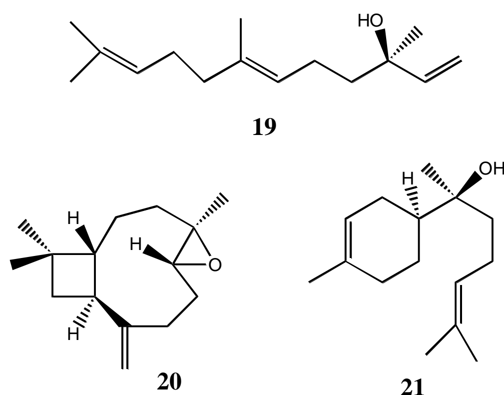
1.5.2.2 Sesquiterpenoids

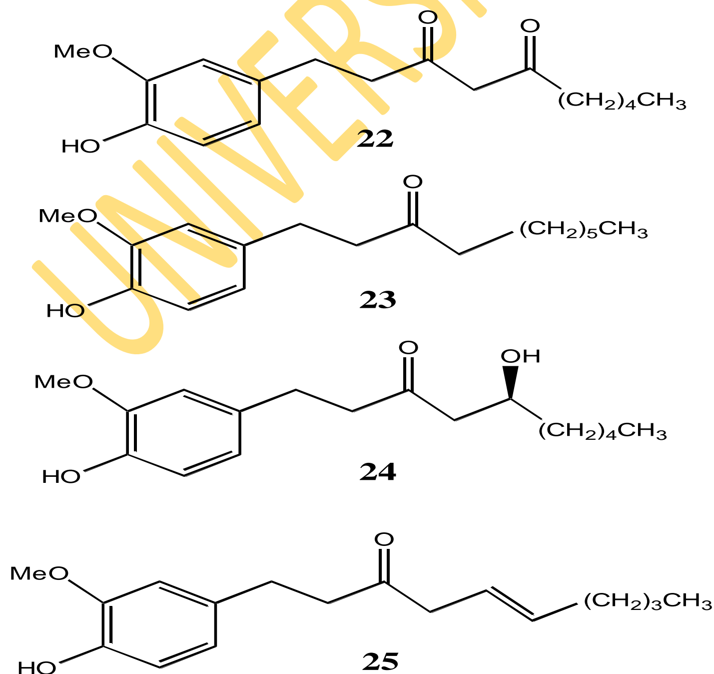
Sesquiterpenoids are rarely found in *Aframomum* species. Up to date only three sesquiterpenoid derivatives have been reported in these species, these are (+)-S-nerolidol (**19**) isolated from *A. sceptrum* (Tomla *et al.,* 2002), and *A. escapum*, (Ayimle *et al.,* 2004), 6,7-epoxy-3(15)-caryophyllene (**20**) and (-)-α-bisabolol (**21**) found in *A arundinaceum*. (Wabo and Tane, n.d).



Stuctures of Sesquiterpenoids

1.5.2.3 Arylalkanoids

Gingerdione (**22**), [6]-paradol (**23**), [6]-gingerol (**24**) and [6]-shogaol (**25**) have been isolated from *A. melegueta*. (Escoubas *et al.,* 1995).



Structures of Arylalkanoids

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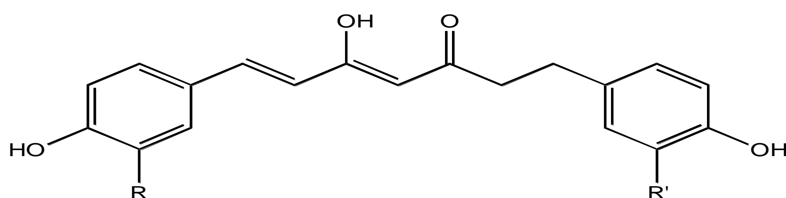
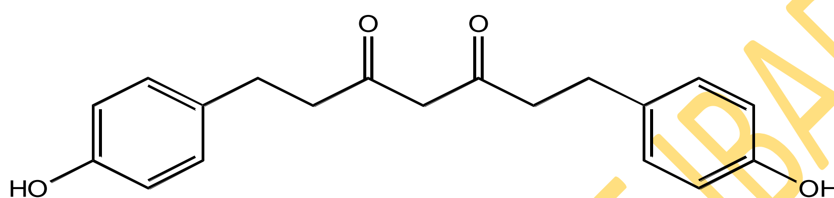
From *A. letestuianum* (Kamnaing *et al.,*2003), four diarylheptanoids were isolated: (4Z,6E)-5-hydroxy-1,7-bis(4-hydroxyphenyl)hepta-4,6-dien-3-one (**26**), letestuianin A (**27**), B (**28**) and C (**29**).



26 R = R' = H

27 R = H, R' = Me

28 R = OMe, R' = H



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Stucture of Arylalkanoids

1.5.2.4 Flavonoids

Flavonoids, as diterpenoids, are commonly found in *Aframomum* species. Both of them can be considered as chemotaxonomic markers of the genus. Eight flavonoids have been reported in five different species of the genus. Kaempferol-3,7,4'-trimethylether (**30**), quercetin-3,7,3',4'-tetramethylether (**31**), quercetin-3,7,4'-trimethylether (**32**) were isolated from *A. giganteum*. (Vidari *et al.,* 1991), 3-Acetoxy-5,7,4'-trihydroxyflavone (**33**) has been isolated from *A. letestuianum,* (Kamnaing *et al.,*2003) , *A. sceptrum* (Tomla *et al.,* 2002), *A. pruinosum* (Ayafor *et al.,* 1981), and *A. handburyii*. (Tsopmo *et al.,*1996), 3-Acetoxy-5,4'-dihydroxy-7-methoxyflavone (**34**) and 3,5-dihydroxy-7,4'-dimethoxyflavanone (**35**) were found in *A. letestuianum, A. pruinosum* and *A. handburyii*. (Kamnaing *et al.,*2003;Tsopmo *et al.,*1996) 3-Acetoxy-5,7-dihydroxy-4'-methoxyflavone (**36**) was reported in *A. pruinosum* and *A. handburyii*. (Tsopmo *et al.,*1996; Ayafor *et al.,* 1981,) Finally 3,5,7,4'-tetrahydroxyflavone (**37**) was obtained from *A. sceptrum*. (Tomla *et al.,* 2002).

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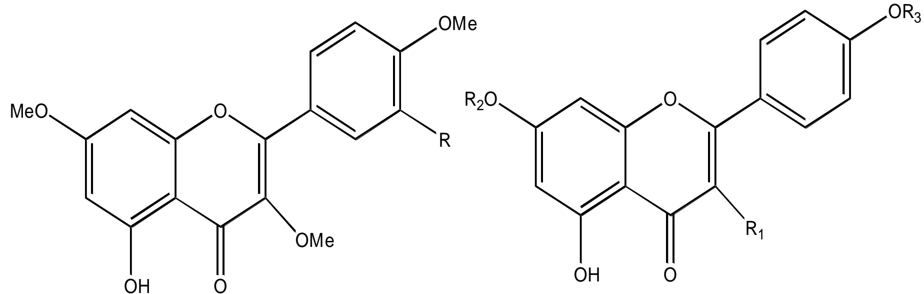

30 R = H

31 R = OMe

32 R = OH

33 R_1 = OAc, R_2 = R_3 = H

34 R_1 = OAc , R_2 = Me, R_3 = H

35 R_1 = OH, R_2 = R_3 = Me

36 R_1 = OAc, R_2 = H, R_3 = Me

37 R_1 = OH, R_2 = R_3 = H

Structures of Flavonoids

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CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 EXPERIMENTAL MATERIALS

2. 1.1 EXPERIMENTAL ANIMALS

Twenty five Male albino rats weighing between 150-250 g were purchased from Covenant Farm, Ibadan, Nigeria and were housed in the experimental animal facility Department of Biochemistry, University of Ibadan. They were fed with commercial rat pellets (Vita Feeds, Mokola, Ibadan, Nigeria) and water *ad libitum*.

2.1.2 PLANT MATERIALS

The *Aframomum longiscapum* seed was purchased from Bodija market Ibadan, Oyo State, Nigeria and identified at the herbarium in the Department of Botany and Microbiology, University of Ibadan.

2.1.3 APPARATUS/INSTRUMENT

(A) Materials for the preparation of test substances.

Beakers, electric blender, measuring cylinders, sieve and test substance bottles.

(B) Materials for the administration of Test substances

Oral intubators, hand gloves, syringe and needles.

(C) Materials for sacrificing Animals, collection of blood and Serum, liver samples, caudal epididymis and bones marrow smear.

Dissecting set, Dissecting board, disposable syringe, disposable needles, precleaned slides, heparinized tubes, cotton wool, sample bottles, Pasteur pipette and centrifuge.

(D) Materials for staining.

Cover glasses, rectangular tray, staining jar, precleaned slides and petri dishes.

(E) Apparatus for scoring of slides.

Nikon Compound microscope and Tally counter.

(F) Apparatus for Liver Function Test.

Micropipette, timer, test-tubes, test-tube rack, incubator, spectrophotometer.

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2.1.4 CHEMICALS/REAGENTS FOR ANALYSIS

Double Distilled water was obtained from the Department of Biochemistry Laboratory, University of Ibadan.

Sodium arsenite ($NaAsO_2$; BDH chemicals Ltd poole England) was dissolved in distilled water and administered at a dose of 2.5 mg/kg body weight corresponding to $1/10^{th}$ of the oral LD_{50} of the salt (Preston *et al.,* 1987).

2.2 EXPERIMENTAL DESIGN

The albino rats were allowed to acclimatise for two weeks before the commencement of study.

The animals for the experiment were divided into five groups (I-V) of five rats per group;

Group I: Served as the control and were fed with rat pellets and distilled water daily.

Group II: A dose of 2.5mg/kg body weight Sodium arsenite dissolved in water (corresponding to $1/10^{th}$ of the oral LD_{50} of the salt) according to Preston *et al.,* 1987.

Group III: A dose of 2.5mg/kg body weight Sodium arsenite (corresponding to $1/10^{th}$ of the oral LD_{50} of the salt) according to Preston, *et al,* 1987 and 3% ethanol(v/v) were given to the rats through oral intubations once a week for a period of five weeks after the initiation of the experiment.

Group IV: The aqueous extract of dry seed of *Aframomum longiscapum* 122.5 mg/kg body weight (Solomon and Babatunde, 2007), was given after every three days through oral intubations for a period of five weeks.

Group V : A dose of 2.5mg/kg body weight Sodium arsenite (corresponding to $1/10^{th}$ of the oral LD_{50} of the salt) according to Preston, *et al,* 1987 and 3% ethanol(v/v) were given to the rats through oral intubations once a week for a period of five weeks after the initiation of the experiment, and aqueous extract of dry seed of *Aframomum longiscapum* 122.5 mg/kg body weight (Solomon and Babatunde, 2007) after every third day for a period of five weeks .

Twenty-four hours after the last exposure to sodium arsenite and ethanol all the rats were sacrificed by cervical dislocation.

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2.3 METHODS

2.3.1 PREPARATION OF AQUEOUS EXTRACT OF AFRAMOMUM LONGISCAPUM SEED

The *Aframomum longiscapum* seed was grinded using blender and 650g was measured and macerated in 1,300ml of distilled water for 72hrs. The aqueous extract was concentrated in a rotary evaporator, lyophilized and thereafter preserved for further use. The yield was 8.62g (1.32615%).

2.3.2 PREPARATION OF REAGENTS

1.15% KCl.

1.15 g of potassium chloride (KCl) was dissolved in distilled water and the volume made up to 100 mL. This was used as a rinsing buffer during the harvesting of liver samples.

10% FORMAL SALINE.

8.5g of sodium chloride was dissolved in 900ml distilled water and made up to 1000ml with formalin.

0.4M NaOH

16g of NaOH was dissolved in distilled water and the volume made up to 1000mL. It was used for ALT and AST assays.

0.01M PHOSPHATE BUFFER (PH 6.8)

0.71 g of disodium hydrogen phosphate (Na_2HPO_4) and 0.68 g of potassium dihydrogen phosphate (KH_2PO_4) was dissolved in water and made up to 1000 ml, and then the pH was adjusted to 6.8. This was used to prepare 5% Giemsa and to rinse the slides while washing.

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5% GIEMSA STAIN

5g of Giemsa was dissolved in phosphate buffer (pH 6.8) and the volume made up to 100 mL. It was used for micronucleus assay.

0.04% COLCHICINE

0.04g Colchicine was dissolved in distilled water and the volume made up to 100 ml. It was used to inject the rats (1ml/100g body weight) two hours prior to the sacrifice to arrest the metaphase.

0.4% MAY-GRUENWALD STAIN 1

0.4ml of May-Gruenwald stain was dissolved and made up to 100ml with absolute methanol.

0.4% MAY-GRUENWALD STAIN 2

1:1 dilution was made using stain 1 and distilled water. They were used in staining of slides.

2.3.3 THE MICRONUCLEI ASSAY

Micronucleus refers to a chromosomal fragment which is lagging in anaphase for various reasons. It is therefore not incorporated into the daughter nuclei at the time of cellular division. In telophase stage of cell division, this material becomes inserted into one of the daughter cells, and either fuses with the main nucleus or forms one or several secondary nuclei (if any). These are significantly smaller than the main nucleus and hence are called micronuclei. They can be observed in any dividing cell population, which has lost some chromosomal fragments (Heddle and Salamone, 1981).

2.3.4 DETECTION OF MICRONUCLEUS

The micronuclei assay is developed for detection of in vivo chromosomal breakage more conveniently than the traditional cytogenetic methods (Heddle, 1973). It has been used to detect *in vivo* genetic activity in bone marrow cells (Sai *et al*., 1992). The

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micronucleus test has also been employed to detect *in vitro* chromosomal aberration (Sasaki *et al*., 1980). The majority of the micronuclei are found in the polychromatic erythrocyte (PCE) cells and this offers an advantage for the use of the micronucleus assay for screening mutagens (Von Le De Bur and Schmid, 1973).

Positive result with the micronucleus test is just an indication of chromosomal damage; it does not conclude the tested agent as a mutagen or carcinogen. However, most studies have proved the agent being tested as mutagenic or carcinogenic agents (Salamon *et al.,* 1980).

2.3.5 PRINCIPLE OF MICRONULEUS ASSAY

The principle of the micronucleus assay is based on the fact that polychromatic erythrocyte (PCE) cells have a staining property that is different from the normal mature erythrocyte (normocyte). The polychromatic staining property results from the presence of ribosomal RNA 24 hours prior to the formation of the cell. As PCEs develop into mature erythrocytes, they lose the ribosomal RNA and the staining property. In mammals, mature erythrocytes expel their nuclei 8-12 hours after the last mitosis preceding the formation of an erythrocyte. The micronuclei for some reasons are not expelled completely. Micronuclei are not normally found in the circulating erythrocytes in blood because they are filtered out by the spleen (Schalm, 1965).

2.3.5.1 REAGENTS

- Fetal calf serum
- Absolute methanol
- Xylene
- Depex (DPX) mountant.
- 0.4% May Grunwald Stain
- 5% Giemsa Stain.
- 0.01M Phosphate buffer pH 6.8

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2.3.5.2 PREPARATION OF BONE MARROW SMEARS

The method of Schmid (1975) was adopted in the preparation of bone marrow smears. After the rats were sacrificed by cervical dislocation, the femur of each rat was removed and stripped clean of muscle. A pair of scissors was used to make an opening in the iliac region of the femur. A small pin was then introduced into the marrow canal at the epiphyseal end. As the pin was pushed inside the canal, the marrow exuded through the hole at the iliac end.

The marrow was placed into a slide and a drop of fetal calf serum was added to the smear using a Pasteur pipette. The whole content was mixed to become homogeneous by using a clean edge of another slide. The homogeneous mixture was then spread on the slide as a smear and allowed to dry.

2.3.5.3 FIXING AND STAINING OF SLIDES

The stepwise procedure includes:

- Fixing the slides in methanol for 5 minutes.
- Air drying to remove the methanol.
- Staining 0.4% May Gruenwald stain 1
- Then in stain 2 May Gruenwald
- Air drying of the slides
- Staining in 5% Giemsa for at least 30 minutes.
- Rinsing in phosphate buffer for about 30 seconds.
- Rinsing in distilled water.
- Air drying of the slides.
- Fixing the slides in xylene for 20 minutes
- Air drying of the slides.
- Mounting in DPX (a natural mountant) with cover slips.

2.3.5.4 SCORING OF THE SLIDES

The fixed cells on the slides were viewed under light microscope to detect the presence of micronucleated polychromatic erythrocytes. Tally counter was used for

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scoring. The slides were first screened at medium magnification to get suitable regions for scoring. PCEs and micronuclei stain blue while normal mature erythrocytes stain red.

2.3.6 PREPARATION OF SERUM SAMPLES FOR ENZYME ASSAYS

The heart of each rat was located underneath the thoracic cavity and blood sample was collected through retro-orbital bleeding. Each blood sample was collected from each and every experimental rat, and then put into a new clean plain bottle. The plain bottles had been labeled appropriately according to the administered test substances. All blood samples collected were later centrifuged at 3,000g for 30 minutes giving rise to serum (supernatant obtained), which was used for enzyme assays.

2.3.6.1 ALANINE AMINOTRANSFERASE (ALT)

Alanine Aminotransferase (ALT) is an intracellular enzyme involved in amino acid and carbohydrate metabolism. It is present in high concentrations in the liver and muscle. It is involved in the transfer of amino group from alanine to α-Ketoglutarate to form pyruvate and glutamate. The diagnostic implications of this enzyme in the serum include hepatitis and other liver diseases in which the level is often higher than that of AST. Elevated level is also found in metastatic or primary liver neoplasm.

PRINCIPLE (Reitman and Frankel, 1957)

Alanine Aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine.

α-ketoglutarate + L-alanine GPT L-glutamate + pyruvate

REAGENTS

Contents	Initial Concentrations of Solutions
R1. Buffer	
Phosphate buffer	100 mmol/l, pH 7.4
L-alanine	200 mmol/l
A-oxoglutarate	2.0 mmol/l
R2. 2,4-dinitrophenylhydrazine	2.0 mmol/l
Sodium Hydroxide	0.4 mol/l

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PROCEDURE

Wavelength:	Hg 546 nm
Cuvette:	1 cm light path
Incubation Temperature	37°C

Measurement against Reagent blank

Pipette into test tubes	Reagent Blank	Sample
Sample	---	0.1 ml
Solution R1	0.5 ml	0.5 ml
Distilled water	0.1 ml	---
Mix, incubate for exactly 30 min. at 37°C		
Solution R2	0.5 ml	0.5 ml
Mix, allow to stand for exactly 20 min. at 25°C		
Sodium Hydroxide	5.0 ml	5.0 ml

Mixed, read the absorbance of sample (Asample) against the reagent blank after 5 minutes.

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Table 2.1: Standard ALT values

The activity of ALT in the Serum was obtained from the table using the standard curve.

Absorbance	U/l	Absorbance	U/l
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

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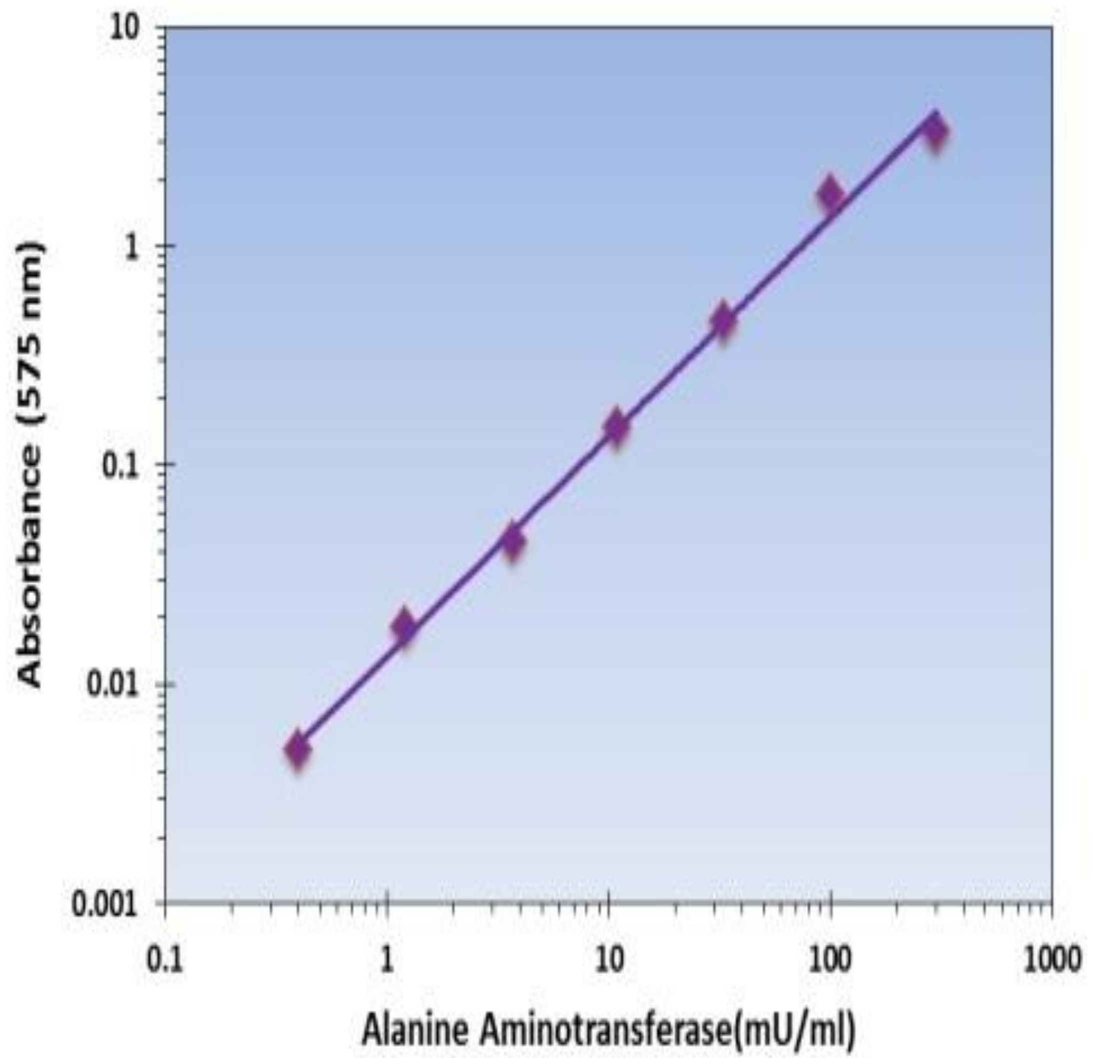

Figure 2.1: Standard ALT curve.

NORMAL VALUES (Schimidt and Schimidt, 1963)

Serum up to 12 U/l

2.3.6.2 ASPARTATE AMINOTRANSFERASE (AST)

Aspartate Aminotransferase (AST) like ALT is an intracellular enzyme involved in amino acid and carbohydrate metabolism. It is present in high concentrations in the liver and muscle. It is involved in the transfer of amino group from aspartate to α-Ketoglutarate to form oxaloacetate and glutamate.

The diagnostic implications of this enzyme in the serum include liver cirrhosis, myocardial infarction (becoming evident 4-8 hours after the onset of pain and peaking after 24-36 hours), muscular dystrophy and paroxysmal myoglobinuria in which the level is often higher than that of AST. As in ALT, elevated level is also found in metastatic or primary liver neoplasm.

PRINCIPLE (Reitman and Frankel, 1957)

AST is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine.

A-oxoglutarate + L-aspartate GOT L-glutamate + Oxaloacetate

REAGENT COMPOSITION

Contents	Initial Concentration of Solutions
R1. Buffer	
Phosphate buffer	100 mmol/l, pH 7.4
L-aspartate	100 mmol/l
A-oxoglutarate	2 mmol/l
R2. 2,4-dinitrophenylhydrazine	2 mmol/l
Sodium Hydroxide Solution	0.4 mol/l

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PROCEDURE

Wavelength:	Hg 546 nm
Cuvette:	1 cm light path
Incubation Temperature	37°C

Measurement against Reagent Blank

Pipette into test tubes:	Reagent Blank	Sample
Sample	---	0.1 ml
Reagent 1	0.5 ml	0.5 ml
Distilled Water	0.1 ml	---
Mix, incubate for exactly 30 min. at 37°C		
Reagent 2	0.5 ml	0.5 ml
Mix, allow to stand for exactly 20 min. at 25°C		
Sodium Hydroxide	5.0 ml	5.0 ml

Mixed, read the absorbance of sample (A_{sample}) against the reagent blank after 5 minutes.

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Table 2.2: Standard AST values.

The activity of ALT in the Serum was obtained from the table using the standard curve.

Absorbance	U/l	Absorbance	U/l
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

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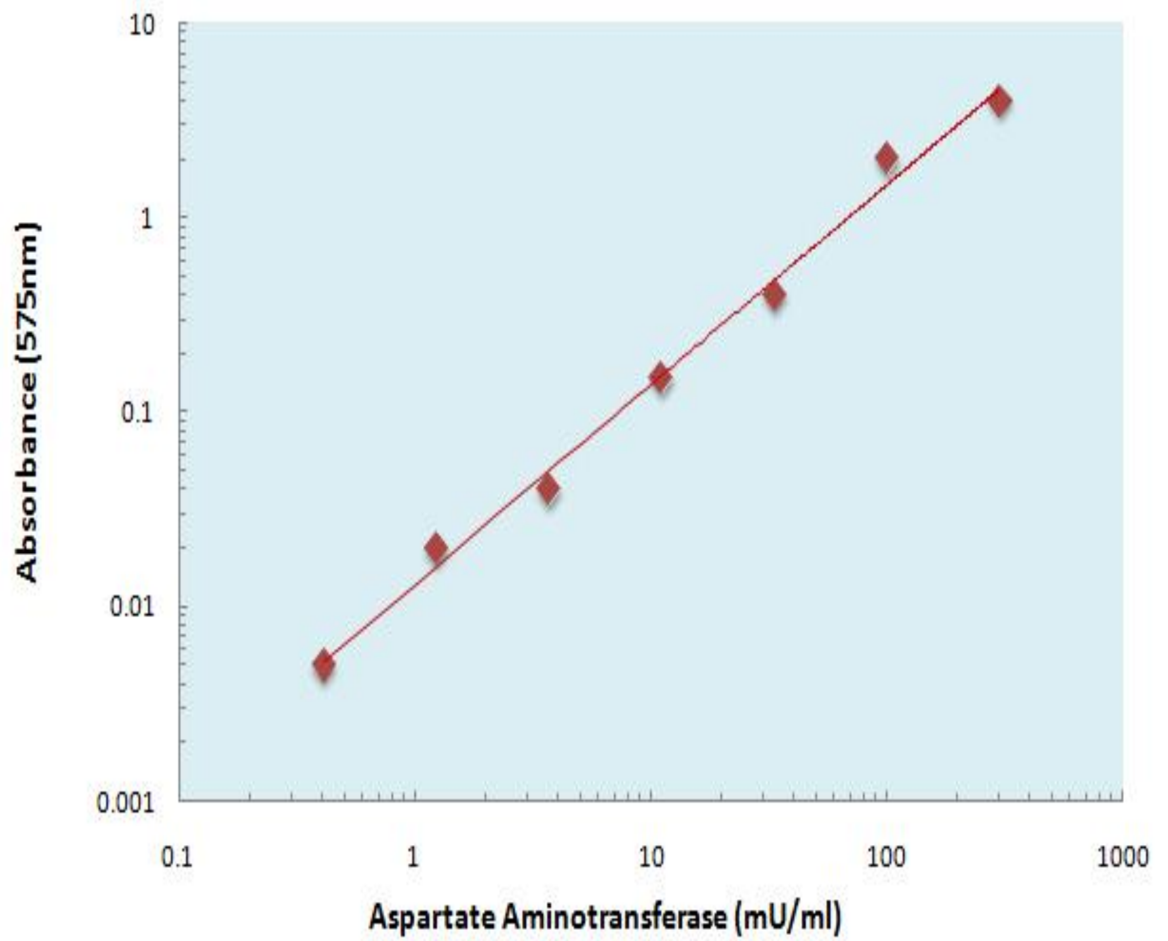

Figure 2.2: Standard AST curve.

NORMAL VALUES (Schmidt and Schmidt, 1963)

Serum up to 12 U/l

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2.3.6.3 ALKALINE PHOSPHATASE (ALP)

COLORIMETRIC METHOD

This is an optimized method according to the recommendations of the Deutsche Gesellschaft fur Klinische Chemie (DGKC, 1972).

PRINCIPLE

p-nitrophenylphosphate + $H_2O \xrightarrow{ALP}$ phosphate + p-nitrophenol

SAMPLE (Englehard *et al*., 1970)

Serum

REAGENT COMPOSITION

Contents	Concentration in the Test
R1a. Buffer	
Diethanolamine buffer	1 mol/l, pH 9.8
$MgCl_2$	0.5 mmol/l
R1b. Substrate	
p-nitrophenylphosphate	10 mmol/l

PROCEDURE

Wavelength:	Hg 405 nm
Cuvette:	1 cm light path
Temperature:	25^oC
Measurement:	against air

The 0.02ml of sample was added into cuvette followed by the 1.0ml of reagent. Mixed , read initial absorbance and started timer simultaneously. Read again after 1,2 and 3 minutes.

CALCULATION

The ALP activity (U/L) = 2760 x ΔA 405 nm/min

NORMAL VALUE IN SERUM

60-170 U/l

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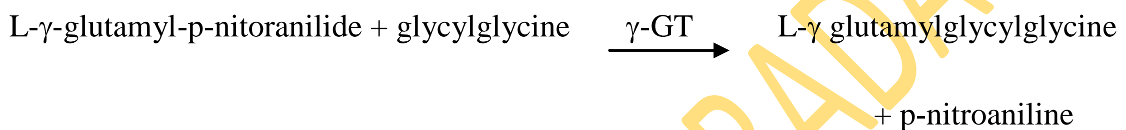
2.3.6.4 GAMMA GLUTAMYL TRANSFERASE

γ-GT activity was determined following the principle described by Szasz (1974).

Principle

Gamma glutamyl transferase (γ-GT) catalyses the transfer of the glutamyl group from glutamyl peptide to an amino acid of another peptide, glylglycine to yield a cleavage product, p-nitro-anilide, which absorbs UV light at 405nm, thus making a direct kinetic determination possible. γ-Glutamyl transferase (E.C. 2.3.2.2.) has been known to be more sensitive and hence more reliable than the serum amino transferases.



PROCEDURE

Wavelength: Hg 405nm

Cuvette: 1cm light path

Temperature: 25^0C, 30^0C, 37^0C

Measurement: against air

The 0.1ml of sample was added into cuvette followed by the 1.0ml of reagent. Mixed , read initial absorbance and started timer simultaneously. Read again after 1,2 and 3 minutes.

CALCULATION

The GGT activity (U/L) = 1158 x ΔA 405 nm/min

NORMAL VALUE IN SERUM

8-38 U/L

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2.3.7 EXTRACTION OF TISSUE SAMPLES

On sacrificed of the experimental rats, their abdominal and thoracic regions were dissected opened exposing the liver. All the livers of experimental animals were harvested one after the other. Part of the harvested liver is cut off and stored in 10% formalin for histopathological examination after being appropriately designated. While the other part of the liver was placed each in a beaker of about 5 ml, chilled KCl in preparation of enzyme assays.

2.3.7.1 HISTOPATHOLOGY

A portion of the liver from each sacrificed animal was excised, blotted and then perfused with potassium chloride (1.15%) in order to remove all traces of haemoglobin which might contaminate the tissues. The liver samples were then preserved and fixed in 10% buffered formalsaline and were processed for paraffin sectioning. Sections of about 5 µm thickness were stained using Haematoxylin and Eosin staining method (Chayen *et al*., 1973), then evaluated by the pathology department of University of Ibadan.

2.3.7.2 HISTOPATHOLOGICAL EXAMINATION

Liver tissues from the animals were immersed in 10% buffered formal-saline. These were left for 24 hours for fixation of the organs after which cross-sections of the organs were cut at 3 mm thickness and placed in a processor overnight. In the processor, the tissues were first placed in 70% alcohol for 2 hours, followed by 90% alcohol for another 2 hours, xylol for 4 hours, and finally, in wax for 5 hours. The tissues were removed, embedded in molten fibro wax and allowed to solidify under a running tap. The tissues were brought out and mounted on wooden blocks and then chilled in ice. Sections of the tissue were cut at a thickness between 3 and 5 mm using the rotary microtome and then allowed to float in 20% alcohol, followed by water at 58°C (an incubator), placed on albumized glass slides and dried on a hot plate at 60°C. The slides so prepared were initially placed in xylol and washed with decreasing concentration of absolute alcohol, 90% alcohol, 80% alcohol and finally, 70% alcohol.

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They were washed in water stained with Cole's haematoxylin, washed again with water, followed by 1% hydrochloric acid, running tap water and rinsed in saturated lithium carbonate. These glass slides were transferred to 1% aqueous solution of eosin for 2 minutes, and washed in a running tap. They were cleaned, mounted on Depex after treatment in absolute alcohol. The slides were finally allowed to dry on the bench at room temperature and then viewed under the microscope.

2.3.8 FERTILITY ANALYSIS OF THE TREATED RATS.

The caudal epididymis was surgically removed, caught opened and a drop of sperm was placed on a slide followed by addition of 2 drops of formal saline. The slide was then viewed under microscope at ×40 objective for motility. The remaining caudal epididymis was dropped in 5.0ml formal saline and change in volume was recorded. The caudal epididymis was removed from the formal saline and transferred into a clean mortar and crushed with pestle. Cover slip was then placed on haemocytometer and later charged with crushed caudal epididymis. It was then viewed under the microscope at ×40 objective for sperm count. However, a little drop of semen was placed on slide followed by addition of haematoxylin and eosin, mixed to smear, viewed at ×40 objective for sperm viability and morphological abnormalities.

2.4 DATA ANALYSIS

The results were expressed as mean ± Standard deviation. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS) software, SPSS Inc., Chicago, Standard version 10.0.1. P-values < 0.05 were considered statistically significant for differences in mean.

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CHAPTER THREE

3.0 RESULTS

3.1.0 **Table 3.1:** Phytochemical screening of aqueous extract of *Aframomum longiscapum* seed

COMPONENTS	RESULTS
Alkaloids	Present
Cardenolides	Present
Anthraquinones	Absent
Saponins	Present
Tannins	Absent
Flavonoids	Present

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3.2.0. BODY WEIGHT RESULT

Table 3.2: Body and Organ weight of the experimental animals (mean ±S.D) and % Weight change

Group	Initial body weight(g)	Final body weight(g)	% Weight change	Liver weight (g)	Relative liver weight(%)
Distilled water only (I)	201±28.81	210±28.50	4.48	6.23±0.51^{b}	3.02±0.53^{b,c}
SA only (II)	205±11.18	215± 13.69	4.88	5.09±0.31^{a,b,c}	2.38 ± 0.21^{a}
SA+ EtOH (III)	235±13.69	260±13.69	10.64	6.44 ± 0.65^{b}	2.47±.15^{a,b}
ALE(IV)	185 13.69	205±20.92	13.51	4.19±0.65^{a,b,c}	2.04±0.16^{a,c}
SA+EtOH+ ALE(V)	180±13.69	175±25.00	-2.78	4.24±0.44^{a,b,c}	2.45±0.29^{a}

a= The mean difference is significant ($p < 0.05$) when compared with group I.

b= The mean difference is significant ($p < 0.05$) when compared with group II.

c= The mean difference is significant ($p < 0.05$) when compared with group III.

SA= Sodium arsenite

EtOH= Ethanol

ALE= *Aframomum longiscapum* extract

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3.3.0 MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES (mPCES) RESULT

Table 3.3: Induction of micronucleated polychromatic erythrocytes (mPCEs) in rat bone marrow cells after exposure to *Aframomum longiscapum,* sodium arsenite and ethanol.

Group	Number of mPCEs/1000 PCEs (Means ± S.D)
Distilled water only (I)	$6.20 \pm 3.69^{b,c}$
SA only (II)	26.50 ± 2.12^{a}
SA+ EtOH (III)	25.80 ± 2.17^{a}
ALE(IV)	$15.40 \pm 5.18^{a,b,c}$
SA+ EtOH+ ALE(V)	$13.60 \pm 4.22^{a,b,c}$

a= The mean difference is significant ($p< 0.05$) when compared with group I.

b= The mean difference is significant ($p< 0.05$) when compared with group II.

c= The mean difference is significant ($p< 0.05$) when compared with group III.

SA= Sodium arsenite

EtOH= Ethanol

ALE= *Aframomum longiscapum* extract

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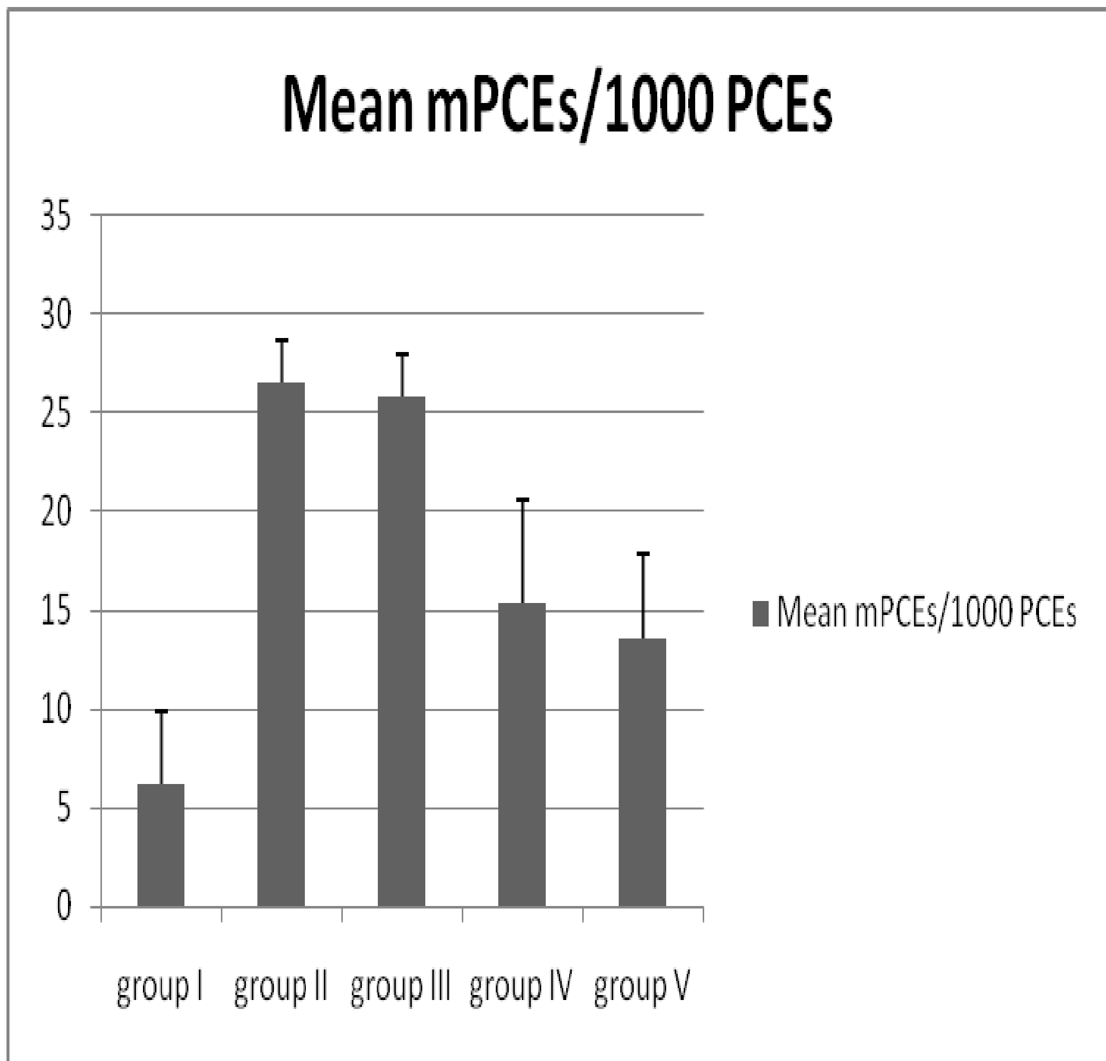

Figure 3.1: Bar charts showing mean mPCEs/1000PCEs ± S.D.

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3.4.0 LIVER ENZYME ASSAYS RESULTS

Table 3.4: Serum level of gamma glutamyl transferase, aspartate aminotransferase, alanine amino transferase and alkaline phosphatase in sera of rats (mean± S.D).

Group	AST (U/l)	ALT (U/l)	ALP (U/l)	γ-GT (U/l)
Distilled water only (I)	47.5 ± 9.19[a,c]	23.12 ± 5.89[c]	236.81±150.18[c]	2.55±1.90[c]
SA only (II)	66 ± 15.56[a,c]	29.87 ± 2.80	432.22 ± 41.38[c]	4.63 ± 1.64[a]
SA+ EtOH (III)	138.60 ± 32.04[a,b]	31.04 ± 2.63[a]	938.95±369.09[a,b]	5.33 ± 2.54[a]
ALE(IV)	92.60±28.63[b,c]	19.84±3.09[b,c]	321.82±220.43[c]	3.01 ± 0.63
SA+ EtOH+ ALE(V)	34.20±37.98[b,c]	21.92±3.97[b,c]	294.22 ± 59.69[c]	3.01±2.10[b,c]

a= The mean difference is significant ($p < 0.05$) when compared with group I.

b= The mean difference is significant ($p < 0.05$) when compared with group II.

c= The mean difference is significant ($p < 0.05$) when compared with group III.

SA= Sodium arsenite

EtOH= Ethanol

ALE= *Aframomum longiscapum* extract

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3.5.0 SPERM QUALITY RESULTS

Table 3.5: Sperm quality in terms of count, motility and viability among the treated rats (mean ± S.D)

Group	Sperm counts	Sperm motility (%)	Sperm viability (%)
Distilled water only (I)	126.00 ± 8.49	90.00 ± 0.00	92.50 ± 3.54
SA only (II)	126 ± 8.48	90 ± 0.00	92.5 ± 3.54
SA+ EtOH (III)	108.00 ± 14.14	70.00 ± 10.00	95.20 ± 3.27
ALE(IV)	85.50 ± 14.85[a]	52.50 ± 15.00[a,b]	93.25 ± 3.95
SA+EtOH+ ALE(V)	85.00 ± 12.73[a,b]	45.00± 20.82[a,b,c]	95.25 ±3.79

a= The mean difference is significant ($p < 0.05$) when compared with group I.

b= The mean difference is significant ($p < 0.05$) when compared with group II.

c= The mean difference is significant ($p < 0.05$) when compared with group III.

SA= Sodium arsenite

EtOH= Ethanol

ALE= *Aframomum longiscapum* extract

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3.6.0 MORPHOLOGICAL ABNORMALITIES RESULTS

Table 3.6: Morphological sperm abnormalities among the treated rats (mean ±S.D).

Group	Total abnormalities (%)
Distilled water only (I)	10.76±1.12
SA only (II)	10.07±1.86
SA+ EtOH (III)	9.99±1.76
ALE(IV)	13.36±2.38
SA+ EtOH+ ALE(V)	13.82±2.84

a= The mean difference is significant ($p< 0.05$) when compared with group I.

b= The mean difference is significant ($p< 0.05$) when compared with group II.

c= The mean difference is significant ($p< 0.05$) when compared with group III.

SA= Sodium arsenite

EtOH= Ethanol

ALE= *Aframomum longiscapum* extract

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3.7.0 HISTOPATHOLOGY RESULTS

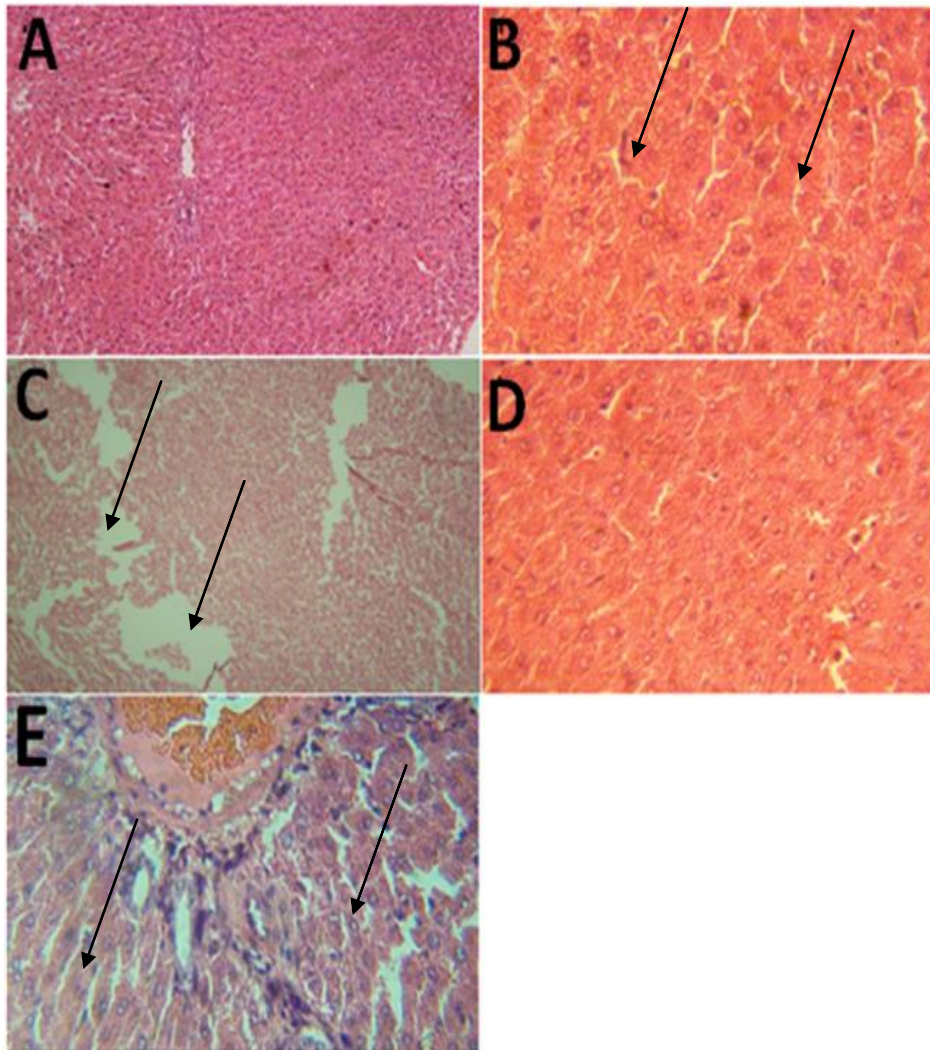

Figure 3.2: Photomicrograph of the liver sections at × 400 magnification

A: Negative control treated rat: No visible lesions seen.

B:Sodium arsenite treated rat: There are multiple areas of extensive vacuolar degeneration of hepatocytes.

C: Sodium arsenite and ethanol treated rat: There is severe hepatic degeneration and necrosis (diffuse).

D: Aqueous extract of *Aframomum longiscapum* seed treated rat: No visible lesion seen.

E: Sodium arsenite + ethanol + Aqueous extract of *Aframomum longiscapum* seed treated rat: Mild hepatic degeneration, mild congestion of the portal vessels and mild mononuclear cellular infiltration

CHAPTER FOUR

4.1 DISCUSSION

Exposure to arsenite has been linked to diverse defects in both experimental animals and in humans (Lonnecker and Daniels, 2001; Tseng *et al*., 2002; Klaassen, 1990; Waalkes *et al*., 2003; Sukla and Pandey, 1984). The liver is an important target organ for arsenic toxicity (Guha, 2005). Arsenic has been claimed to be of clinical utility in the treatment of syphilis, amoebiasis, and certain other tropical diseases (Klaassen, 1990) and also has been used in Fowler solution in the treatment of arthritis (Klassen, 1990), but recently arsenic intoxication in experimental animals has been associated with hepatic tumors (Waalkes *et al*., 2003), the inhibition of testicular steroidogenic function (Sarkar *et al*., 1991),and spermatogenesis (Sukla and Pandey, 1984), as well as with severe metabolic disorders such as diabetes in humans (Lonnecker and Daniels, 2001; Tseng *et al*., 2002). It is known that (SA) can act as co-mutagen due to its ability to inhibit the activities of thiol containing enzymes (Sunderman, 1979), such as DNA ligase (Li and Rossman, 1989) resulting in defective DNA replication, repair, recombination and joining of single- and double-stranded DNA breaks.

This study examined the effect of co-exposure to SA and ethanol in the presence and absence of ALE in male Wistar albino rats. Results from phytochemical studies (Table3.1), show that the plant extract contains flavonoids, alkaloids, cardenolides and saponins with concomitant absence of anthraquinone and tannins. This could be an index of the fact that the aqueous extract of *Aframomum longiscapum* may contain some anti-oxidative properties. The observed increase in body weight of rats fed with ALE (group IV) could be due to the androgenic properties of the plant since androgens possess anabolic activity (Johnson *et al.,*1988). Furthermore, in the group treated with both SA, ethanol and ALE there is also decrease in the liver weight significantly ($p<0.05$) when compared with control. The decrease in body and liver weight of group V rats that was treated with SA, ethanol and ALE might be attributed to the synergistic effects of *Aframomum longiscapum*, ethanol and sodium arsenite. Liver weight of rats treated with SA alone (Table 3.2) decreases significantly ($p<0.05$) as compared with control. However, there was no significant ($p>0.05$) increases in SA and ethanol treated group also as compared with control group. Significant ($p<0.05$) decreases was

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observed in *Aframomum longiscapum* treated group when compare with both group I, II and III. The increase in liver weight observed might be because of the fact that ethanol induces fatty liver with enhanced cholesterol biosynthesis (Jose, 2003). The decrease in liver weight might be due to the fact that the rats were exposed to *Aframomum longiscapum* extract for too long, consequently therefore, its flavonoids anti-oxidative characteristics could have been altered and they tend to become pro-oxidants. There was significant ($p<0.05$) decrease in relative liver weight across all the groups when compare with the control. Similarly, the relative liver weight in *Aframomum longiscapum* treated group decrease significantly ($p<0.05$) as compare with SA, ethanol treated group.

The results of the present study clearly demonstrate that SA alone, SA and ethanol administered, Significantly ($P < 0.05$) induced the formation of micronuclei in the polychromatic erythrocytes (PCEs) of the rat bone marrow cells (Table 3.3). This observation is consistent with earlier observations in our laboratory and those of others on the clastogenic potentials of sodium arsenite in the bone marrow (Odunola, 2003; Odunola *et al.,*2007). In this particular investigation, the induction by sodium arsenite ($1/10^{th}$ of the $LD_{50;}$ 2.5mg/kgbw) and 3% ethanol (v/v) was increased significantly ($p<0.05$) more than 4 fold when compared with the micronucleated polychromatic erythrocytes (mPCEs) induced in the bone marrow of control group. This may be due to the fact that arsenite generates free radicals that can attack DNA leading to chromosomal breakage. In addition acetaldehyde the end product of ethanol metabolism can form DNA adducts and this might explain why arsenite and ethanol can synergistically induced a considerable increase in micronucleated polychromatic erythrocytes (mPCEs). However, from Table 3.3, there was a significant ($p<0.05$) decrease in micronucleated polychromatic erythrocytes (mPCEs) induction in the bone marrow in sodium arsenite, ethanol and aqueous extract of *Aframomum longiscapum* treated rats. More than a 2 fold decrease was observed when compared with groups II and III. This may be attributed to the fact that the *Aframomum longiscapum* possess antioxidant activity that help in scavenging free radicals and reactive oxygen species generated by arsenite and ethanol metabolism. Interestingly, there was also significant ($p<0.05$) increase in micronucleated polychromatic erythrocytes (mPCEs) induction in ALE treated rats when compared with control, but there is no significant ($p<0.05$)

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difference between control and arsenite, ethanol and aqueous extract of *Aframomum longiscapum* treated rats. The increase in micronucleated polychromatic erythrocytes (mPCEs) induction in ALE treated rats might be due to the fact that the rats were exposed to *Aframomum longiscapum* extract for too long, consequently therefore, its flavonoids anti-oxidative characteristics could have been altered and they tend to become pro-oxidants with possibility of having genotoxic effects.

The results obtained from the assessment of the serum activities of ALP, γ-GT, ALT and AST show that SA alone, SA plus ethanol increase serum activity of ALP significantly ($p<0.05$) by about 2 folds and 4 folds respectively compared with the control group. The ALP activity decreased significantly ($p<0.05$) in group V and IV by about 2.5 and 3.5 folds compared with group II and III (Table 3.4). Exposure to sodium arsenite had been shown to induce ALP activity (Chattopady *et al.,*2003). In this study Sodium arsenite and simultaneous administration of arsenic and ethanol also significantly ($p<0.05$) induced serum γ-GT activity by about 2 and 3 folds when compare with the control (Table 3.4). This is an indication of induction of hepatotoxicity and oxidative stress in the hepatocytes. Increased γ-GT activity has been associated with hepatotoxicity, oxidative stress and chromosomal aberrations in cells (Dinari *et al.,*1979; Karmaker *et al.,*1999; Lee *et al.,*2004). In the group treated with SA, ethanol and ALE, the level of γ-GT activity decreased significantly ($p<0.05$) when compare with SA alone, SA plus ethanol treated rats. This reduction may be due to the presence of flavonoids in the extract which have reputable antioxidant properties (Wiseman, 1996). Taken together, SA and SA plus ethanol induced serum activity of ALT (Table 3.4) and AST (Table 3.4) significantly ($p<0.05$) as when compared with control. Interestingly, the level of ALT and AST activity decreased significantly ($p<0.05$) when compared with the SA and ethanol treated rats as well as ALE treated rats. The induction of ALT and AST activity following sodium arsenite has been documented (Mallick *et al.,*2003). Elevation in the level of the enzymes, γ-GT and ALP has emerged as an index of a liver lesion (Lum and Gambino 1972, Friedman et al., 1996).

Result from the sperm quality as an index of reproductive integrity (Table 3.5), shows that the ALE treated group demonstrated a significant ($p<0.05$) decrease in both sperm counts and motility when compared with negative and positive control group. Further

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decrease was also observed in ALE treated rats and arsenic, ethanol and ALE treated rats. This is consistent with findings with other androgen containing compounds (Yewah *et al.,* 2005). However, in terms of viability, it shows no significant ($p>0.05$) difference across all the groups. It means that the *Aframomum* species could also influence male rat fertility positively (Yewah *et al.,* 2005). There were no morphological abnormalities in all the experimental groups in the present study.

The limited histopathological examinations showed no visible lesions in the negative control and the group treated with ALE. Severe hepatic degeneration and necrosis were observed in SA alone treated group, as well as the SA plus ethanol treated group because SA was potentially hepatotoxic. However, mild hepatic degeneration and congestion of the portal vessels observed in ALE, SA plus ethanol treated groups confirmed a protective effect of ALE on SA – induced hepatocytes damage. This support the growing evidence that spices and fruits exhibit protective effects against chemicals and toxins (Prashar et al., 1994, Biswas et al., 1999, Karthikeyan et al., 1999, Rastogi et al., 2007).

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4.2 CONCLUSION

Based on the results of the liver function tests (ALP,ALT, AST and GGT), it could be concluded that the aqueous extract of *Aframomum longiscapum* might have some hepatoprotective effect. The ALE was able to ameliorate the hepatotoxic effect of sodium arsenite and ethanol. The extract also has anticlastogenic properties. In terms of reproductive functions, it can be deduced that the extract has both positive and negative effects on male rat fertility by its ability to increase sperm viability and decrease sperm count and motility.

4.3 RECOMMENDATIONS

1- Molecular mechanisms of the effect of *Aframomum longiscapum* on male fertility should be studied in detail.

2- Inflammatory studies should be carried out on *Aframomum longiscapum* to ascertain whether it could be anti/proinflammatory for its eventual use for cancer chemoprevention.

3- Excessive consumption of *Aframomum longiscapum* should be avoided until the studies are completed.

4- Finally, the active compounds of *Aframomum longiscapum* should be isolated and characterized.

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APPENDIX I

LIST OF BAR CHARTS

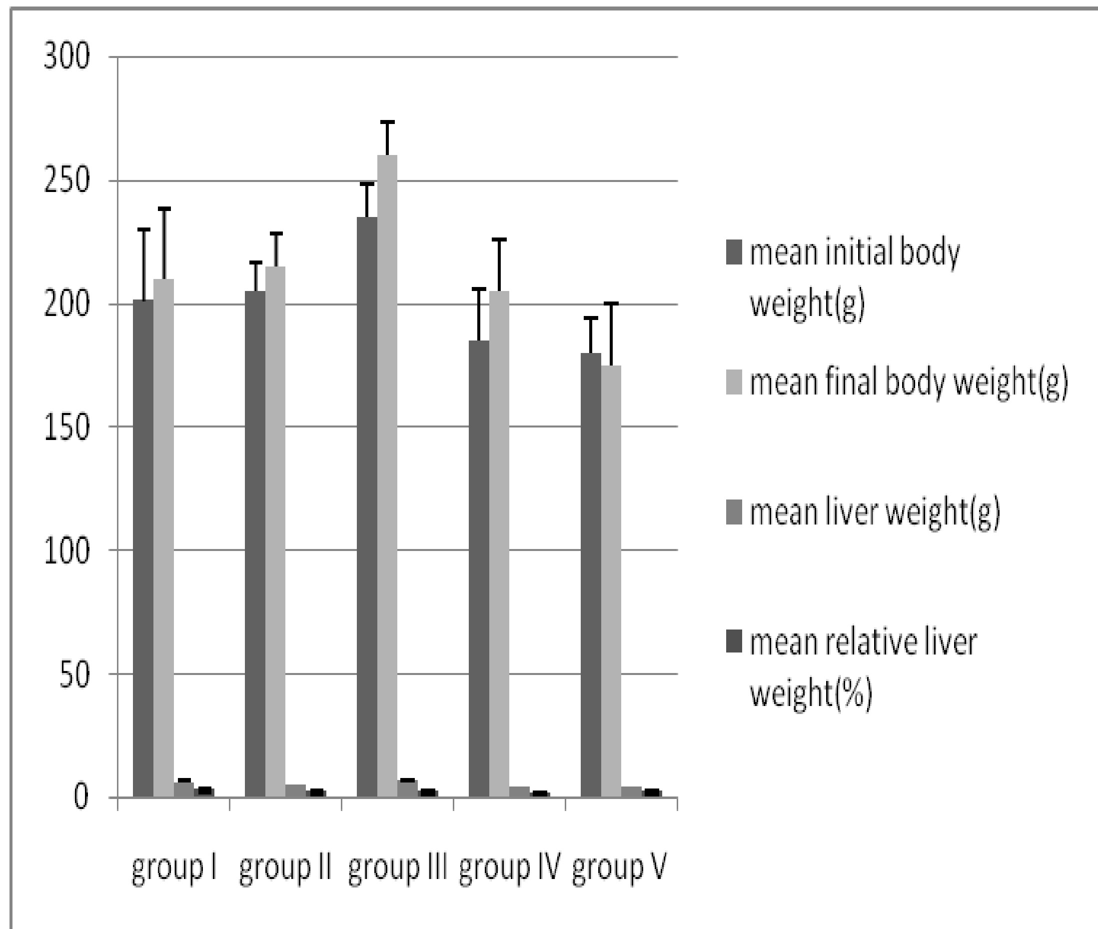

Figure 3a: Bar charts showing mean initial, final body, liver and relative liver weights ± S.D.

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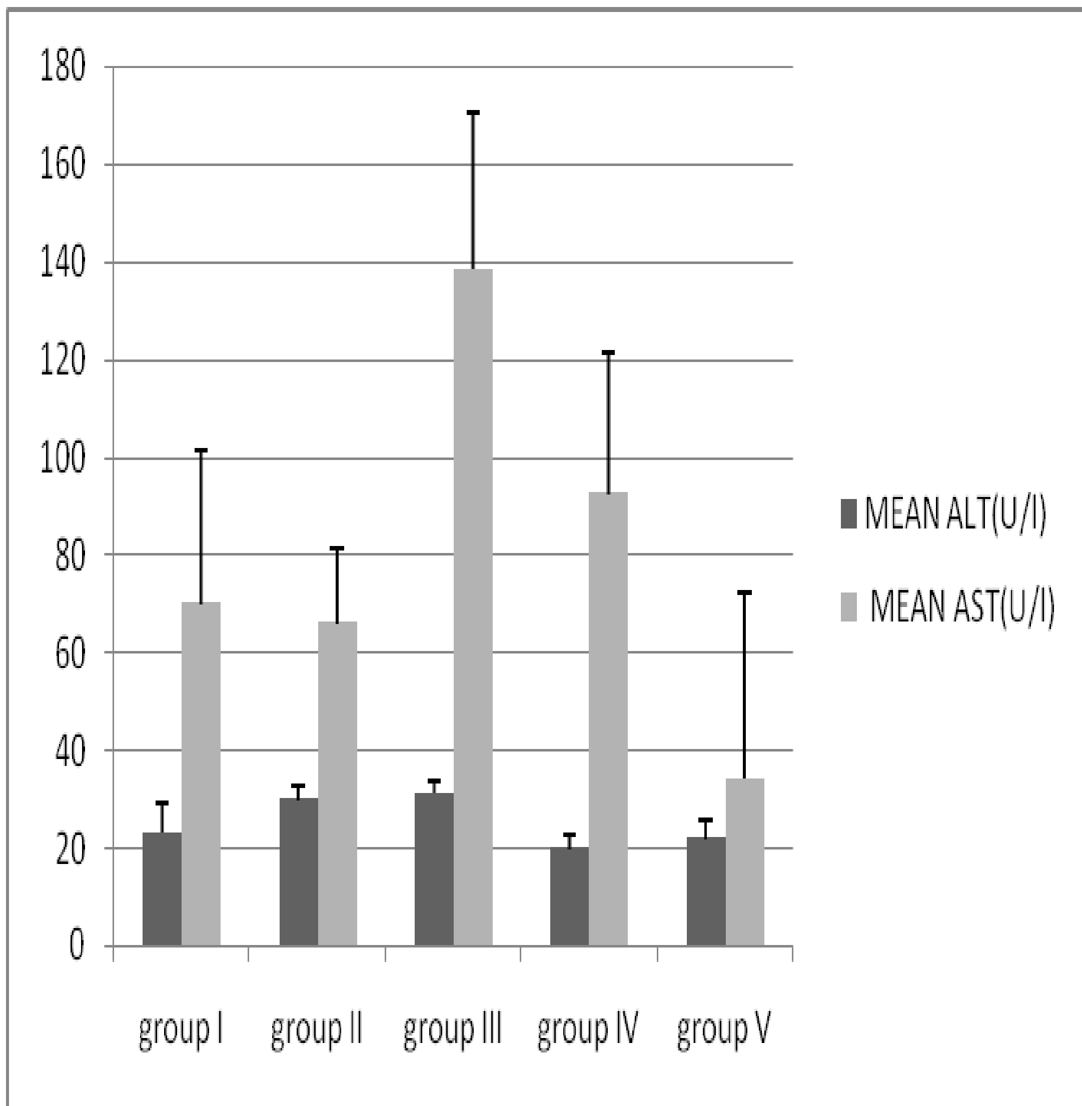

Figure 3b: Bar charts showing mean aspartate and alanine aminotransferases activity (U/l) ± S.D.

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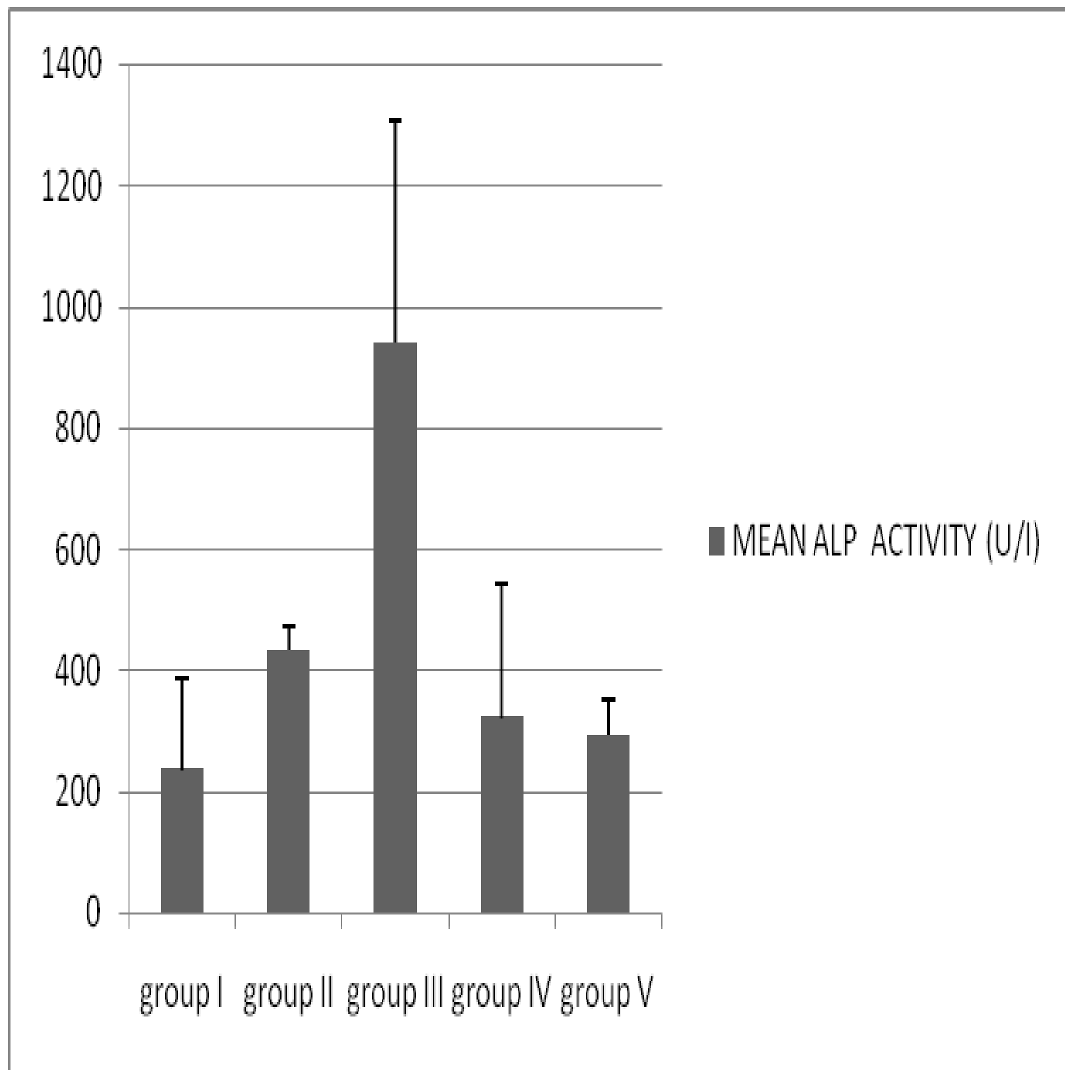

Figure 3c: Bar charts showing mean ALP activity (U/l) ± S.D.

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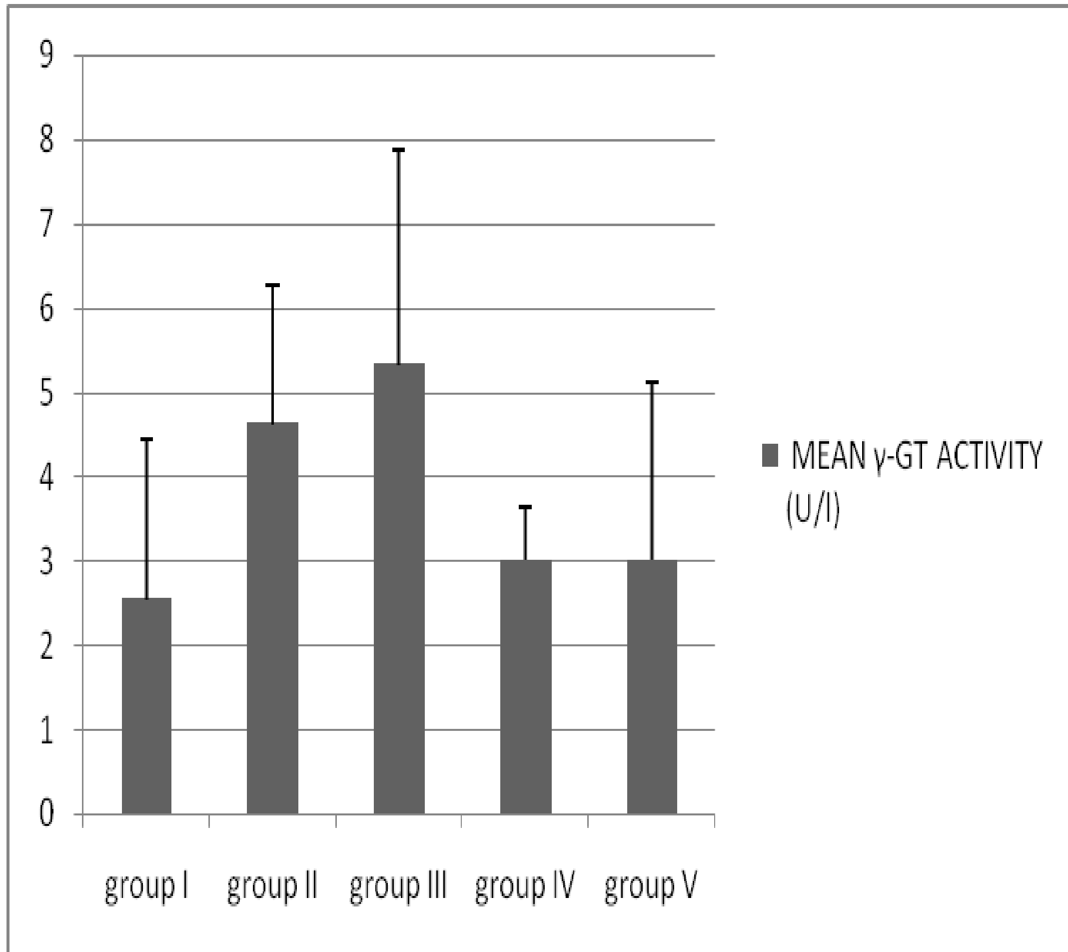

Figure 3d: Bar charts showing mean γ-GT activity (U/l) ± S.D.

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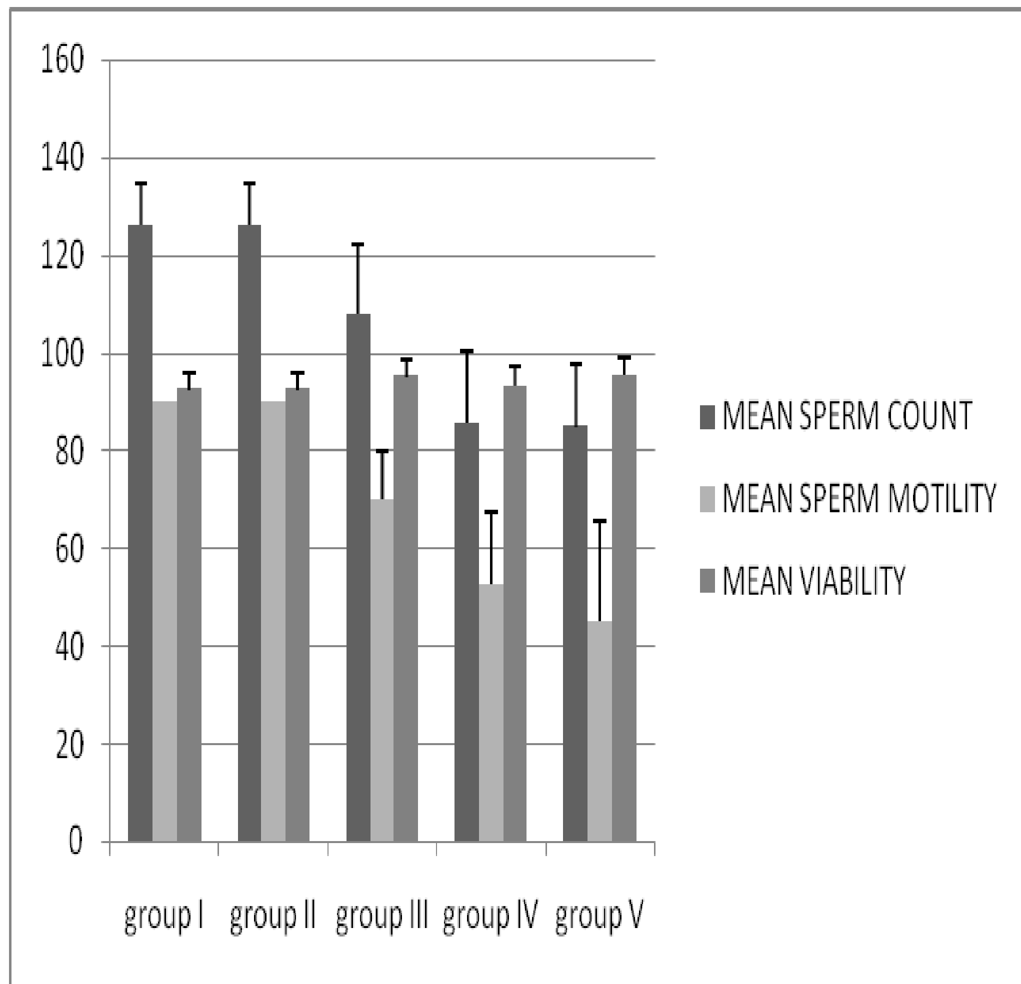

Figure 3e: Bar charts showing mean sperm motility, count and viability ± S.D.

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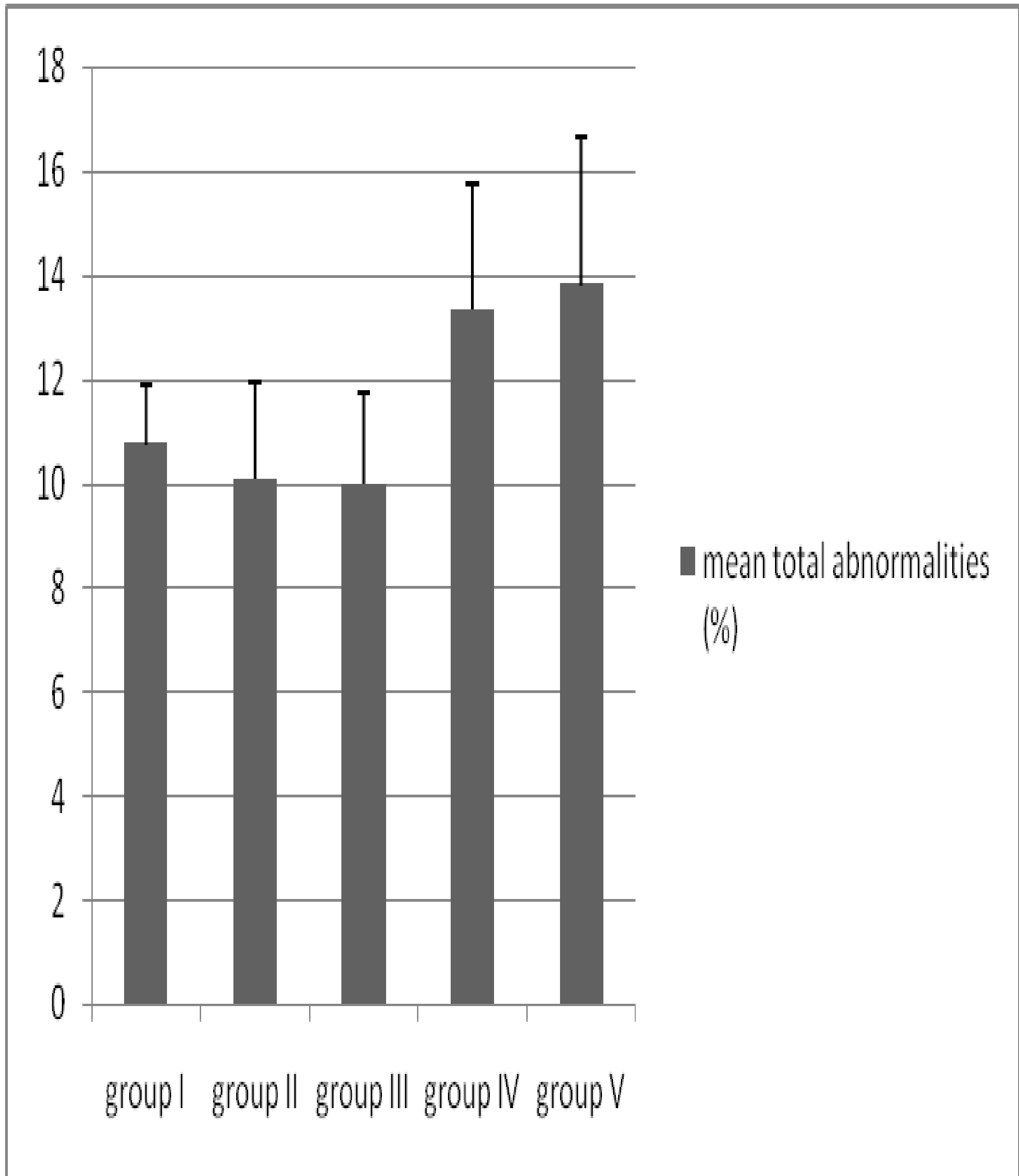

Figure 3f: Bar charts showing mean ± S.D of total morphological characteristics of caudal epididymal spermatozoa.

APPENDIX II

Statistical analysis using one way ANOVA

1= control group

2=$NaAsO_2$ + ethanol treated group

3= aqueous extract of *Afarmomum longiscapum* treated group

4= $NaAsO_2$ + ethanol + aqueous extract of *Afarmomum longiscapum* treated group

ALT activity ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	359.832	3	119.944	7.161	.003
Within Groups	268.000	16	16.750		
Total	627.832	19			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-7.92000*	2.58844	.007	-13.4072	-2.4328
	3.00	3.28000	2.58844	.223	-2.2072	8.7672
	4.00	1.20000	2.58844	.649	-4.2872	6.6872
2.00	1.00	7.92000*	2.58844	.007	2.4328	13.4072
	3.00	11.20000*	2.58844	.001	5.7128	16.6872
	4.00	9.12000*	2.58844	.003	3.6328	14.6072
3.00	1.00	-3.28000	2.58844	.223	-8.7672	2.2072
	2.00	-11.20000*	2.58844	.001	-16.6872	-5.7128
	4.00	-2.08000	2.58844	.433	-7.5672	3.4072
4.00	1.00	-1.20000	2.58844	.649	-6.6872	4.2872
	2.00	-9.12000*	2.58844	.003	-14.6072	-3.6328
	3.00	2.08000	2.58844	.433	-3.4072	7.5672

*. The mean difference is significant at the .05 level.

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AST activity ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28655.350	3	9551.783	8.924	.001
Within Groups	17125.200	16	1070.325		
Total	45780.550	19			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-68.60000*	20.69130	.004	-112.4636	-24.7364
	3.00	-22.60000	20.69130	.291	-66.4636	21.2636
	4.00	35.80000	20.69130	.103	-8.0636	79.6636
2.00	1.00	68.60000*	20.69130	.004	24.7364	112.4636
	3.00	46.00000*	20.69130	.041	2.1364	89.8636
	4.00	104.40000*	20.69130	.000	60.5364	148.2636
3.00	1.00	22.60000	20.69130	.291	-21.2636	66.4636
	2.00	-46.00000*	20.69130	.041	-89.8636	-2.1364
	4.00	58.40000*	20.69130	.012	14.5364	102.2636
4.00	1.00	-35.80000	20.69130	.103	-79.6636	8.0636
	2.00	-104.40000*	20.69130	.000	-148.2636	-60.5364
	3.00	-58.40000*	20.69130	.012	-102.2636	-14.5364

*. The mean difference is significant at the .05 level.

ALP activity ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1626039	3	542013.061	10.278	.001
Within Groups	843740.6	16	52733.788		
Total	2469780	19			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-702.14400*	145.23607	.000	-1010.0307	-394.2573
	3.00	-85.00800	145.23607	.567	-392.8947	222.8787
	4.00	-57.40800	145.23607	.698	-365.2947	250.4787
2.00	1.00	702.14400*	145.23607	.000	394.2573	1010.0307
	3.00	617.13600*	145.23607	.001	309.2493	925.0227
	4.00	644.73600*	145.23607	.000	336.8493	952.6227
3.00	1.00	85.00800	145.23607	.567	-222.8787	392.8947
	2.00	-617.13600*	145.23607	.001	-925.0227	-309.2493
	4.00	27.60000	145.23607	.852	-280.2867	335.4867
4.00	1.00	57.40800	145.23607	.698	-250.4787	365.2947
	2.00	-644.73600*	145.23607	.000	-952.6227	-336.8493
	3.00	-27.60000	145.23607	.852	-335.4867	280.2867

*. The mean difference is significant at the .05 level.

γ-GT activity ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23.601	3	7.867	2.114	.139
Within Groups	59.539	16	3.721		
Total	83.140	19			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-2.77920*	1.22003	.037	-5.3655	-.1929
	3.00	-.46320	1.22003	.709	-3.0495	2.1231
	4.00	-.46320	1.22003	.709	-3.0495	2.1231
2.00	1.00	2.77920*	1.22003	.037	.1929	5.3655
	3.00	2.31600	1.22003	.076	-.2703	4.9023
	4.00	2.31600	1.22003	.076	-.2703	4.9023
3.00	1.00	.46320	1.22003	.709	-2.1231	3.0495
	2.00	-2.31600	1.22003	.076	-4.9023	.2703
	4.00	.00000	1.22003	1.000	-2.5863	2.5863
4.00	1.00	.46320	1.22003	.709	-2.1231	3.0495
	2.00	-2.31600	1.22003	.076	-4.9023	.2703
	3.00	.00000	1.22003	1.000	-2.5863	2.5863

*. The mean difference is significant at the .05 level.

mPCEs/1000 PCEs ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	823.632	3	274.544	28.015	.000
Within Groups	147.000	15	9.800		
Total	970.632	18			

Multiple Comparisons

Dependent Variable: VAR00001

	(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1.00	2.00	-18.30000*	2.10000	.000	-22.7760	-13.8240
		3.00	-5.50000*	2.10000	.019	-9.9760	-1.0240
		4.00	-6.90000*	2.10000	.005	-11.3760	-2.4240
	2.00	1.00	18.30000*	2.10000	.000	13.8240	22.7760
		3.00	12.80000*	1.97990	.000	8.5799	17.0201
		4.00	11.40000*	1.97990	.000	7.1799	15.6201
	3.00	1.00	5.50000*	2.10000	.019	1.0240	9.9760
		2.00	-12.80000*	1.97990	.000	-17.0201	-8.5799
		4.00	-1.40000	1.97990	.490	-5.6201	2.8201
	4.00	1.00	6.90000*	2.10000	.005	2.4240	11.3760
		2.00	-11.40000*	1.97990	.000	-15.6201	-7.1799
		3.00	1.40000	1.97990	.490	-2.8201	5.6201

*. The mean difference is significant at the .05 level.

Sperm count ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3016.983	3	1005.661	8.023	.004
Within Groups	1378.750	11	125.341		
Total	4395.733	14			

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Multiple Comparisons

Dependent Variable: VAR00001

LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	27.00000*	9.36689	.015	6.3836	47.6164
	3.00	40.25000*	9.69565	.002	18.9100	61.5900
	4.00	44.00000*	9.69565	.001	22.6600	65.3400
2.00	1.00	-27.00000*	9.36689	.015	-47.6164	-6.3836
	3.00	13.25000	7.51022	.105	-3.2799	29.7799
	4.00	17.00000*	7.51022	.045	.4701	33.5299
3.00	1.00	-40.25000*	9.69565	.002	-61.5900	-18.9100
	2.00	-13.25000	7.51022	.105	-29.7799	3.2799
	4.00	3.75000	7.91647	.645	-13.6740	21.1740
4.00	1.00	-44.00000*	9.69565	.001	-65.3400	-22.6600
	2.00	-17.00000*	7.51022	.045	-33.5299	-.4701
	3.00	-3.75000	7.91647	.645	-21.1740	13.6740

*. The mean difference is significant at the .05 level.

Sperm motility ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1585.000	3	528.333	1.042	.412
Within Groups	5575.000	11	506.818		
Total	7160.000	14			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-20.00000	18.83541	.311	-61.4565	21.4565
	3.00	-2.50000	19.49650	.900	-45.4115	40.4115
	4.00	5.00000	19.49650	.802	-37.9115	47.9115
2.00	1.00	20.00000	18.83541	.311	-21.4565	61.4565
	3.00	17.50000	15.10193	.271	-15.7391	50.7391
	4.00	25.00000	15.10193	.126	-8.2391	58.2391
3.00	1.00	2.50000	19.49650	.900	-40.4115	45.4115
	2.00	-17.50000	15.10193	.271	-50.7391	15.7391
	4.00	7.50000	15.91883	.647	-27.5371	42.5371
4.00	1.00	-5.00000	19.49650	.802	-47.9115	37.9115
	2.00	-25.00000	15.10193	.126	-58.2391	8.2391
	3.00	-7.50000	15.91883	.647	-42.5371	27.5371

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Sperm viability ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	18.533	3	6.178	.469	.710
Within Groups	144.800	11	13.164		
Total	163.333	14			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-2.70000	3.03555	.393	-9.3812	3.9812
	3.00	-.75000	3.14209	.816	-7.6657	6.1657
	4.00	-2.75000	3.14209	.400	-9.6657	4.1657
2.00	1.00	2.70000	3.03555	.393	-3.9812	9.3812
	3.00	1.95000	2.43385	.440	-3.4069	7.3069
	4.00	-.05000	2.43385	.984	-5.4069	5.3069
3.00	1.00	.75000	3.14209	.816	-6.1657	7.6657
	2.00	-1.95000	2.43385	.440	-7.3069	3.4069
	4.00	-2.00000	2.56551	.452	-7.6466	3.6466
4.00	1.00	2.75000	3.14209	.400	-4.1657	9.6657
	2.00	.05000	2.43385	.984	-5.3069	5.4069
	3.00	2.00000	2.56551	.452	-3.6466	7.6466

Tailless head ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.089	3	.030	.432	.734
Within Groups	.754	11	.069		
Total	.843	14			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.19206	.21901	.399	-.2900	.6741
	3.00	.22825	.22669	.336	-.2707	.7272
	4.00	.09875	.22669	.672	-.4002	.5977
2.00	1.00	-.19206	.21901	.399	-.6741	.2900
	3.00	.03619	.17560	.840	-.3503	.4227
	4.00	-.09331	.17560	.606	-.4798	.2932
3.00	1.00	-.22825	.22669	.336	-.7272	.2707
	2.00	-.03619	.17560	.840	-.4227	.3503
	4.00	-.12950	.18510	.499	-.5369	.2779
4.00	1.00	-.09875	.22669	.672	-.5977	.4002
	2.00	.09331	.17560	.606	-.2932	.4798
	3.00	.12950	.18510	.499	-.2779	.5369

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Headless tail ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.611	3	.537	4.416	.024
Within Groups	1.580	13	.122		
Total	3.191	16			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.66315*	.23389	.014	-1.1684	-.1579
	3.00	-.68000*	.24654	.016	-1.2126	-.1474
	4.00	-.80950*	.24654	.006	-1.3421	-.2769
2.00	1.00	.66315*	.23389	.014	.1579	1.1684
	3.00	-.01685	.23389	.944	-.5221	.4884
	4.00	-.14635	.23389	.542	-.6516	.3589
3.00	1.00	.68000*	.24654	.016	.1474	1.2126
	2.00	.01685	.23389	.944	-.4884	.5221
	4.00	-.12950	.24654	.608	-.6621	.4031
4.00	1.00	.80950*	.24654	.006	.2769	1.3421
	2.00	.14635	.23389	.542	-.3589	.6516
	3.00	.12950	.24654	.608	-.4031	.6621

*. The mean difference is significant at the .05 level.

Rudimentary tail ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.609	3	.203	4.936	.019
Within Groups	.493	12	.041		
Total	1.102	15			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.44175*	.14338	.010	-.7541	-.1294
	3.00	-.37475*	.14338	.023	-.6871	-.0624
	4.00	-.49975*	.14338	.005	-.8121	-.1874
2.00	1.00	.44175*	.14338	.010	.1294	.7541
	3.00	.06700	.14338	.649	-.2454	.3794
	4.00	-.05800	.14338	.693	-.3704	.2544
3.00	1.00	.37475*	.14338	.023	.0624	.6871
	2.00	-.06700	.14338	.649	-.3794	.2454
	4.00	-.12500	.14338	.400	-.4374	.1874
4.00	1.00	.49975*	.14338	.005	.1874	.8121
	2.00	.05800	.14338	.693	-.2544	.3704
	3.00	.12500	.14338	.400	-.1874	.4374

*. The mean difference is significant at the .05 level.

Bent tail ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.556	3	2.519	5.741	.010
Within Groups	5.704	13	.439		
Total	13.260	16			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-1.24433*	.44433	.015	-2.2042	-.2844
	3.00	-1.67175*	.46836	.003	-2.6836	-.6599
	4.00	-1.68225*	.46836	.003	-2.6941	-.6704
2.00	1.00	1.24433*	.44433	.015	.2844	2.2042
	3.00	-.42742	.44433	.354	-1.3873	.5325
	4.00	-.43792	.44433	.342	-1.3978	.5220
3.00	1.00	1.67175*	.46836	.003	.6599	2.6836
	2.00	.42742	.44433	.354	-.5325	1.3873
	4.00	-.01050	.46836	.982	-1.0223	1.0013
4.00	1.00	1.68225*	.46836	.003	.6704	2.6941
	2.00	.43792	.44433	.342	-.5220	1.3978
	3.00	.01050	.46836	.982	-1.0013	1.0223

*. The mean difference is significant at the .05 level.

Curved tail ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.049	3	2.683	6.388	.007
Within Groups	5.460	13	.420		
Total	13.509	16			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-1.35715*	.43474	.008	-2.2964	-.4179
	3.00	-1.61450*	.45826	.004	-2.6045	-.6245
	4.00	-1.80800*	.45826	.002	-2.7980	-.8180
2.00	1.00	1.35715*	.43474	.008	.4179	2.2964
	3.00	-.25735	.43474	.564	-1.1966	.6819
	4.00	-.45085	.43474	.319	-1.3901	.4884
3.00	1.00	1.61450*	.45826	.004	.6245	2.6045
	2.00	.25735	.43474	.564	-.6819	1.1966
	4.00	-.19350	.45826	.680	-1.1835	.7965
4.00	1.00	1.80800*	.45826	.002	.8180	2.7980
	2.00	.45085	.43474	.319	-.4884	1.3901
	3.00	.19350	.45826	.680	-.7965	1.1835

*. The mean difference is significant at the .05 level.

Bent mid-pierce tail ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.629	3	1.876	3.975	.033
Within Groups	6.137	13	.472		
Total	11.766	16			

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Multiple Comparisons

Dependent Variable: VAR00001

LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-1.12854*	.46091	.029	-2.1243	-.1328
	3.00	-1.37200*	.48584	.014	-2.4216	-.3224
	4.00	-1.49850*	.48584	.009	-2.5481	-.4489
2.00	1.00	1.12854*	.46091	.029	.1328	2.1243
	3.00	-.24346	.46091	.606	-1.2392	.7523
	4.00	-.36996	.46091	.437	-1.3657	.6258
3.00	1.00	1.37200*	.48584	.014	.3224	2.4216
	2.00	.24346	.46091	.606	-.7523	1.2392
	4.00	-.12650	.48584	.799	-1.1761	.9231
4.00	1.00	1.49850*	.48584	.009	.4489	2.5481
	2.00	.36996	.46091	.437	-.6258	1.3657
	3.00	.12650	.48584	.799	-.9231	1.1761

*. The mean difference is significant at the .05 level.

Curved mid-pierce tail ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.594	3	2.198	5.209	.014
Within Groups	5.486	13	.422		
Total	12.079	16			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-1.24675*	.43576	.013	-2.1881	-.3054
	3.00	-1.67775*	.45933	.003	-2.6701	-.6854
	4.00	-1.38025*	.45933	.010	-2.3726	-.3879
2.00	1.00	1.24675*	.43576	.013	.3054	2.1881
	3.00	-.43100	.43576	.341	-1.3724	.5104
	4.00	-.13350	.43576	.764	-1.0749	.8079
3.00	1.00	1.67775*	.45933	.003	.6854	2.6701
	2.00	.43100	.43576	.341	-.5104	1.3724
	4.00	.29750	.45933	.528	-.6948	1.2898
4.00	1.00	1.38025*	.45933	.010	.3879	2.3726
	2.00	.13350	.43576	.764	-.8079	1.0749
	3.00	-.29750	.45933	.528	-1.2898	.6948

*. The mean difference is significant at the .05 level.

Looped tail ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.026	2	.013	.171	.845
Within Groups	.837	11	.076		
Total	.863	13			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2.00	3.00	-.10830	.18507	.570	-.5156	.2990
	4.00	-.06010	.18507	.751	-.4674	.3472
3.00	2.00	.10830	.18507	.570	-.2990	.5156
	4.00	.04820	.17448	.787	-.3358	.4322
4.00	2.00	.06010	.18507	.751	-.3472	.4674
	3.00	-.04820	.17448	.787	-.4322	.3358

Initial body weight ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9253.750	3	3084.583	7.512	.002
Within Groups	6570.000	16	410.625		
Total	15823.750	19			

Multiple Comparisons

Dependent Variable: VAR00001

LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-34.00000*	12.81601	.017	-61.1687	-6.8313
	3.00	16.00000	12.81601	.230	-11.1687	43.1687
	4.00	21.00000	12.81601	.121	-6.1687	48.1687
2.00	1.00	34.00000*	12.81601	.017	6.8313	61.1687
	3.00	50.00000*	12.81601	.001	22.8313	77.1687
	4.00	55.00000*	12.81601	.001	27.8313	82.1687
3.00	1.00	-16.00000	12.81601	.230	-43.1687	11.1687
	2.00	-50.00000*	12.81601	.001	-77.1687	-22.8313
	4.00	5.00000	12.81601	.702	-22.1687	32.1687
4.00	1.00	-21.00000	12.81601	.121	-48.1687	6.1687
	2.00	-55.00000*	12.81601	.001	-82.1687	-27.8313
	3.00	-5.00000	12.81601	.702	-32.1687	22.1687

*. The mean difference is significant at the .05 level.

Final body weight ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	18625.000	3	6208.333	12.040	.000
Within Groups	8250.000	16	515.625		
Total	26875.000	19			

Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-50.00000*	14.36141	.003	-80.4448	-19.5552
	3.00	5.00000	14.36141	.732	-25.4448	35.4448
	4.00	35.00000*	14.36141	.027	4.5552	65.4448
2.00	1.00	50.00000*	14.36141	.003	19.5552	80.4448
	3.00	55.00000*	14.36141	.001	24.5552	85.4448
	4.00	85.00000*	14.36141	.000	54.5552	115.4448
3.00	1.00	-5.00000	14.36141	.732	-35.4448	25.4448
	2.00	-55.00000*	14.36141	.001	-85.4448	-24.5552
	4.00	30.00000	14.36141	.053	-.4448	60.4448
4.00	1.00	-35.00000*	14.36141	.027	-65.4448	-4.5552
	2.00	-85.00000*	14.36141	.000	-115.4448	-54.5552
	3.00	-30.00000	14.36141	.053	-60.4448	.4448

*. The mean difference is significant at the .05 level.

Liver weight ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22.506	3	7.502	23.249	.000
Within Groups	5.163	16	.323		
Total	27.669	19			

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Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.21400	.35926	.560	-.9756	.5476
	3.00	2.03000*	.35926	.000	1.2684	2.7916
	4.00	1.98800*	.35926	.000	1.2264	2.7496
2.00	1.00	.21400	.35926	.560	-.5476	.9756
	3.00	2.24400*	.35926	.000	1.4824	3.0056
	4.00	2.20200*	.35926	.000	1.4404	2.9636
3.00	1.00	-2.03000*	.35926	.000	-2.7916	-1.2684
	2.00	-2.24400*	.35926	.000	-3.0056	-1.4824
	4.00	-.04200	.35926	.908	-.8036	.7196
4.00	1.00	-1.98800*	.35926	.000	-2.7496	-1.2264
	2.00	-2.20200*	.35926	.000	-2.9636	-1.4404
	3.00	.04200	.35926	.908	-.7196	.8036

*. The mean difference is significant at the .05 level.

Relative liver weight ANOVA

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.391	3	.797	7.716	.002
Within Groups	1.653	16	.103		
Total	4.044	19			

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Multiple Comparisons

Dependent Variable: VAR00001
LSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.54075*	.20327	.017	.1098	.9717
	3.00	.97263*	.20327	.000	.5417	1.4035
	4.00	.57001*	.20327	.013	.1391	1.0009
2.00	1.00	-.54075*	.20327	.017	-.9717	-.1098
	3.00	.43187*	.20327	.050	.0010	.8628
	4.00	.02925	.20327	.887	-.4017	.4602
3.00	1.00	-.97263*	.20327	.000	-1.4035	-.5417
	2.00	-.43187*	.20327	.050	-.8628	-.0010
	4.00	-.40262	.20327	.065	-.8335	.0283
4.00	1.00	-.57001*	.20327	.013	-1.0009	-.1391
	2.00	-.02925	.20327	.887	-.4602	.4017
	3.00	.40262	.20327	.065	-.0283	.8335

*. The mean difference is significant at the .05 level.

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